Stability of llama heavy chain antibody fragments under extreme conditions

Stabiliteit van zware keten antilichaam fragmenten van lama's in extreme condities

(met een samenvatting in het Nederlands)

Proefschrift

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Cold-hearted orb that rules the night
Removes the colours from our sight,
Red is grey and yellow white
But we decide which is right
And which is an illusion.

The Moody Blues

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Chapter 1

General Introduction

General introduction

Immune system

The immune system is of great importance for the defence of our body against invasion of pathogens, like viruses and bacteria. The importance is best illustrated by the result of a defect in the immune system, for instance after infection with a virus impairing the immune system, like HIV (the AIDS virus) of which we all know the dramatic consequences.

The immune system discriminates two defence mechanisms, which are integrated to form a complex defence system. The innate or native immunity, which is the first line of defence, and adaptive immunity, which adapts to and develops an immune response to infection or intrusion of the body.

The innate immunity consists of several basic mechanisms to inhibit a rapid and not very selective first line of defence. To these mechanisms belong the mucosal layers of the intestine, epithelia, and the inflammatory reaction as non-selective barriers. Also, phagocytic cells, the complement system, and a variety of other effector cells, which are coordinated by cytokines. The main difference with adaptive immunity is that innate immunity responds in a similar way to repetitive infection. Adaptive immunity on the other hand adapts its response during repeated exposure and evolves a highly specific response to the specific intruder.

The adaptive immune system has several defence mechanisms. One mechanism is known as cell-mediated immunity and is particularly effective against invaded viruses, parasites and cancer cells. T-cells mediate the recognition of these abnormal cells in the body and destroy them by activation of a series of effector cells, like killer T-cells. Another mechanism is called humoral immunity and is particularly directed against extracellular bacteria and viruses. Here, B-cells and helper T-cells recognize foreign molecules resulting in the secretion of specific antibodies in the blood. Antibodies specifically bind to the foreign molecules (antigens) in the body. Although the antigens (*anti*body *gen*erators) need to be large (>10kDa) to provoke an immune response, the antibody binds to only a small part of the antigen, the epitope. Binding of the antibodies to these antigens causes the destruction of the foreign molecule by macrophages or complement system.

Antibodies are also referred to as immunoglobulins (Ig), because of the globular structure seen in all subdomains of the antibodies. Five classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM are found. The different classes have different structures according to their function in the body. IgM and IgD are in membrane-bound form on naive B-cells. They have a major role in the antibody response in the blood.

IgA is responsible for the first line of defence outside the blood stream, for instance in saliva or the mucosal layers of the intestine. Two subclasses of IgA have been found, IgA1 and IgA2, of which IgA1 is most abundant. IgE is less abundant and has a function in the defence against parasites and plays a role in allergy. IgG is relatively abundant and is especially important for the secondary immune response. Four isotypic subclasses of IgG are now known, IgG1, IgG2, IgG3, and IgG4, which are different in structure and therefore the different subclasses have specific functions. They can all develop high affinity binding to their antigen and have a lot of secondary biological functions. This makes IgG the working ant of the immune system.

Structure of antibodies

IgG comprises four polypeptide chains. Two identical heavy (H) chains and two identical light (L) chains. These chains are linked by disulphide bonds to form a functional antibody. The peptide chains are made up of related domains. The light chain consists of two domains, a constant domain and a variable domain. The heavy chain is more complex and consists of several domains depending on the isotype. IgG heavy chains consist of a variable domain and the first constant domain combined by a flexible linker to two constant domains (see figure 1). Soluble IgD heavy chains resemble IgG. IgA and IgM also resemble IgG, but IgA can form dimers, and IgM can form pentamers, both linked by an extra domain or J-chain. IgE heavy chains consist of five domains, with CH4 as an extra domain.

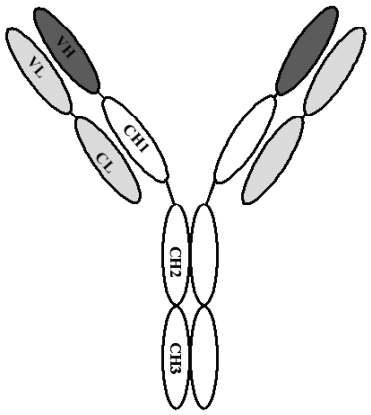

Figure 1 Schematic representation of an IgG. In light gray the light chains with a variable domain (VL) and a constant domain (CL). In dark gray the variable domain of the heavy chain (VH) and in white the constant domains of the heavy chain (CH).

Folding of the polypeptide chains begins in the ER and occurs independently for heavy and light chains, and thus before assembly. As a quality control system the BiP protein is associated to the first constant domain of the heavy chain (CH1) (Knarr, 1995), preventing aggregation and causing retention in the ER (Hendershot, 1987). Association of the light chain to the heavy chain results in displacement of the BiP protein and in the secretion of the complete antibody from the ER via the Golgi to the extracellular space (Hendershot, 1990). In this process, formation of disulphide bridges results in a stable antibody as shown in figure1. This shows the dual role of BiP, first preventing aggregation of the heavy chains and at the same time presenting them to the light chain.

This assembly of heavy and light chain results in two adjacent variable domains, interacting to form the antigen binding domains. Within these variable domains three regions of high variability could be recognized, the complementarity determining regions (CDRs). These loops are in direct contact with the antigen and determine the specificity of the antibody. These binding loops are grafted on a double β-sheet 'sandwich' structure typical for antibodies, called the framework.

Llama heavy chain antibodies

The antibodies as described above are present in all animals and will be referred to as conventional antibodies. However, for camelids (Hamers-Casterman, 1993), wobbegong or nurse sharks (Greenberg, 1995) and spotted ratfish (Rast, 1998) it has been shown that they also posses a structurally different kind of antibodies next to conventional antibodies. These antibodies are composed of only two identical heavy polypeptide chains, resembling the heavy chains of conventional antibodies and are therefore referred to as heavy chain antibodies (HCAbs).

Llamas, dromedaries and camels in the family of the *Camilidae* are the only species of the suborder of the *Tylopodia*. Heavy chain antibodies have not been reported in closely related taxonomic suborders, like *Ruminantia* (giraffe, sheep) or *Suiformes* (swines, hippopotamuses) of the order of *Artiodactyla*, to which the *Tylopodia* belong (Conrath, 2003) (see figure 2). This shows that the heavy chain antibodies have evolved a relatively short time ago (16-18 million years) (Conrath, 2003). The camelid heavy chain antibodies have evolved independently from those of the ratfish and nurse shark and are not a remnant of evolution but emerged from the conventional antibodies (Nguyen, 2002).

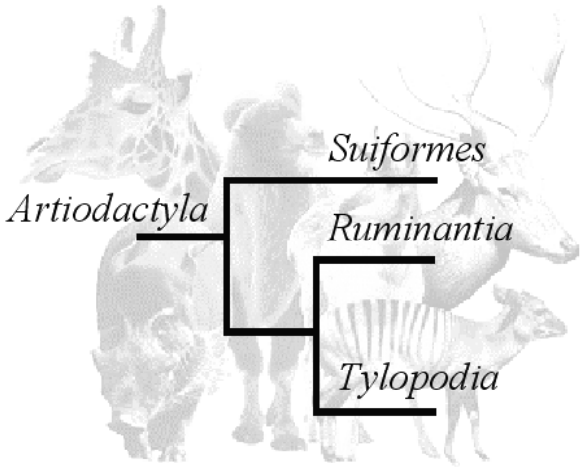

Figure 2 Phylogenetic tree of the order *Artiodactyla.*

The heavy chains of the HCAbs lack the first constant region (Ch1). The CH1 genes in camelids have a mutation at the canonical splicing site, which is responsible for the loss of the first constant domain in the heavy chain antibodies. The BiP protein can no longer attach to the heavy chains, to combine them with the light chains. This might be the crucial step in the emergence of heavy chain antibodies (Nguyen, 2002). The two remaining constant domains are coupled to the variable domain by a flexible linker or hinge (see figure 3). This variable region is, by the lack of the light chain, also the only domain responsible for binding to the antigen. This **v**ariable domain of the **h**eavy chain of **h**eavy chain antibodies is called VHH (see figure 3).

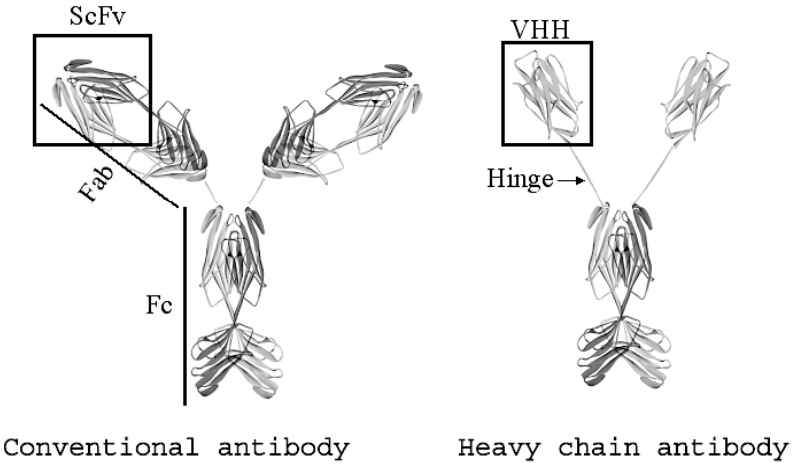

Figure 3 Representation of a conventional antibody and a heavy chain antibody.

Engineered antibody fragments

An antibody can be divided into two antigen binding fragments (Fab) and a crystallizable fragment (Fc) or effector fragment (see figure 3). The Fab fragments of conventional antibodies consist of two variable domains and two constant domains, which can still bind specifically to an antigen. An even smaller binding unit consists of only the two variable domains (VH-VL) combined by a linker, the single chain variable fragment (ScFv) (see figure 3). VHHs derived from heavy chain antibodies are the smallest naturally occurring binding domains.

The benefits of small binding units have been recognized and are now emerging rapidly. For instance lipocalins, which are small secreted proteins with certain immunomodulatory effects are now being used as an alternative for antibodies (Skerra, 2001). These single domain 'anticalins' are able to bind a wide diversity of molecules, resembling recombinant antibody fragments (Schlehuber, 2002).

The llama VHH domain is transcribed from a separate set of genes (Nguyen, 2000) and VHHs thus have unique properties compared to the variable domain of the heavy chain of conventional antibodies (VH) (Harmsen, 2000). Four residues, Val37, Gly44, Leu45 and Trp47 (Kabat numbering is used throughout (Kabat, 1991)), are highly conserved in VHs. These residues are in the hydrophobic patch which interacts with the VL, and are constitutively substituted in the VHHs into Phe/Tyr37, Glu44, Arg45 and Phe/Leu/Gly47 (reviewed in Muyldermans, 2001). These substitutions make the hydrophobic interface more hydrophilic and are specific for VHHs, and are therefore recognized as hallmarks for VHHs. They render the VHH more soluble, thereby showing adaptation to their single domain status. This adaptation is necessary for compensation of the loss of stability induced by the lack of VL. Since local unfolding is usually the start of denaturation induced by negative influences, like high temperature and denaturants, the VHH is susceptible for these influences. Local unfolding in the single domain can easily result in global unfolding, where the paired domains of VH and VL can stabilize each other.

Several features have been shown to enlarge the diversity of the VHH repertoire to compensate for the lack of the VL domain in the binding to the antigen. A large subset of VHH specific germline genes, together with a longer CDR3, and more residues involved in binding of the antigen lead to an enhanced repertoire. The longer CDR3 not only leads to a increase in binding repertoire, but also gives the VHH a higher structural complexity, and

therefore have more abilities in binding the antigen. The long CDR3 is reported to efficiently protrude into the active site clefts of enzymes (Transue, 1998; Lauwereys, 1998), form cavities for hapten binding (Spinelli, 2000) and show lateral recognition of the antigen (Spinelli, 2001). CDR3 alone was shown able to bind specific to and with high affinity to the antigen (Desmyter, 2001). In general more residues are involved in antigen binding, not only as a result of a longer CDR3, but also because of more incidences of framework residues involved in antigen binding (Spinelli, 2001). Together with a high diversity of specific VHH genes (Nguyen, 2000), this leads to a high diversity of antigen binding capacity.

Antibodies in biology and the industry

Antibodies are key players in the immune response in the body. Their major function, the specific and high affinity binding to any given antigen, make them well appreciated in practically all disciplines of biology. Detection of protein bands on a Western blot with specific antibodies is used in all the molecular biology and biochemistry labs, and nowadays even antibody arrays (Zhu, 2000; Haab, 2003) are just two applications in which antibodies are well established.

In the industry the applications using antibody technology are rising rapidly as well. Antibodies are used in several diagnostic tests to detect small amounts of drugs or hormones, e.g. monoclonal antibodies to human chorionic gonadotropin (HCG) are used in OTC pregnancy test kits (Porter, 1988). In medicine, antibodies are used as fusion proteins, to function as a carrier to target specific effector substances to a specific location, the so-called ‘magic bullet’ approach (Joosten, 2003). Cancer therapy is one of the promising applications for the use of antibodies with this ‘magic bullet’ approach (Boleti, 1995). Antibodies have also proven their usefulness in radioimmunodetection and radioimmunotherapy of cancer (Funaro, 2000; Goodwin, 2001; Roovers, 2001), in which the small, easily penetrating VHHs might have an advantage over conventional antibodies (Cortez-Retamozo, 2002). Oral or intravenous administration of antibodies are used for the treatment of various diseases, like Huntington’s disease by inhibition of aggregation of huntingtin (Heiser, 2000). The direct inhibition of rotavirus by oral administration of antibodies is another example of an application for antibodies in therapeutics (Hilpert, 1987). A drawback recognized by Heiser *et al* (2000), Hilpert *et al* (1987) and many other

researchers is the decreased functionality of antibodies *in vivo*. They may be degraded by proteases, have a poor blood–brain permeability, are unable to reach their target protein, or are simply not functional *in vivo*, because of physical limitations of the antibodies.

In this respect the VHHs are now emerging and more and more researchers recognize the advantages and possibilities of these small natural binding blocks. Inhibiting aggregation of human lysozyme by binding of VHHs to decrease fibril and plaque formation seen in amyloid diseases (Dumoulin, 2003) and the detection and characterization of other diseases, like another aggregation associated disease, oculopharyngeal muscular dystrophy (OPMD) (van Koningsbruggen, 2003) shows that VHHs can be very useful for therapeutic purposes. The direct monovalent inhibition of enzymes and viruses shows the potential of these antibody fragments (Stijlemans, 2003; Dekker, 2003; Van der Vaart, in preparation), even across the blood-brain barrier (Muruganandam, 2002) or in the gastrointestinal tract (Dolk, in preparation). In electron microscopy the easily penetratable VHHs can be used to detect and localize proteins, like the baseplate protein ORF18 of lactococcal bacteriophage p2, which is involved in adsorption to cell surface receptors (Ledeboer, 2002; de Haard, accepted for publication), and in other applications like immunoprecipitation, and immunoaffinity purification they have proven their functionality (Verheesen, 2003).

One of the reasons for the interest of the industry in VHHs is that the single domain allows production in microorganisms, like the yeast *Saccharomyces cerevisiae*. The high levels of production and the subsequent purification is very efficient, which enables cheap production of large quantities of VHHs (Frenken, 2000; Thomassen, 2002).

One of the most prominent advantages of VHHs over conventional antibodies is their performance at high temperatures. Van der Linden reported on the excellent properties of VHHs at high temperatures, showing VHHs can endure incubations of two hours at up to 90 ºC. Some VHHs were even able to bind to their antigen at 90 ºC (Van der Linden, 1999). Perez showed that temperature denatured unfolding was reversible and functional VHHs were present after cooling down (Perez, 2001). This reversibility of unfolding was also shown at 80 ºC, induced by the antigen (Dolk, in preparation). This shows that the stability of the antibody-antigen complex can result in the refolding of the VHH. High temperatures up to 70°C necessary for pasteurisation of food products and even higher temperatures up to 120°C for sterilization of diagnostic tools with VHHs belong to the possibilities with

VHHs. Whereas conventional antibodies would loose significant functionality, because of irreversible unfolding. These observations explain the apparent high intrinsic stability of the VHHs. Altogether the easy cloning, cheap production and high stability make VHHs very useful for a wide range of applications.

Definition of stability

Since stability of VHHs is the major topic of this thesis, it is important to have a clear definition of what is called stability. The definition of stability in the dictionary is: "the quality or attribute of being firm and steadfast" and the definition of stableness is: "the quality of being steady or securely and immovably fixed in place". In biology stableness or stability might mean just the opposite. To explain this contradiction and to clarify what stability means in this thesis, we have to consider the differences in determination of the stability of a protein. In this respect, we can distinguish two kinds of stability, intrinsic stability and functional stability. The intrinsic stability of a protein in a certain environment is simply the difference in Gibbs free energy, ΔG, between the folded and the unfolded state of the protein defined by the enthalpy, the entropy and the temperature ($\Delta G = \Delta H - T\Delta S$).

On the other hand, the functional stability will be defined as the ability of the protein to function under the given conditions. This definition of stability does not only consider the intrinsic stability of a protein but also the kinetic stability of a protein. A protein that is completely unfolded at high temperature, but for instance by reversible folding, is fully functionality, will be considered stable by the latter definition, but should be considered thermo-unstable. Aggregation, proteolysis and chemical degradation are factors that will decrease stability of proteins denaturing reversible. In this case flexibility and reversibility will be parameters that define stability. This could be considered the opposite of the definition given by the dictionary: "immovably fixed in place".

In this thesis the term stability will be used as functional stability under the given conditions. An increase in stability implicates that the protein is more functional at a certain temperature or concentration of denaturant, compared to another protein. It could also implicate that the protein is able to maintain the same functionality at higher temperatures or at higher concentrations of denaturants compared to another protein.

Phage display and selection for stability

To select the VHH with the desired properties from a large collection of VHH, phage display is performed (Hoogenboom, 1998). Phage display uses bacteria and bacterial viruses, known as bacteriophage, to select antigen specific antibody fragments (in our case VHHs). This technology combines the VHH displayed on bacteriophage particles to the DNA encoding this VHH, which is present in the phage. One of the strengths of this method is this coupling of phenotype to genotype.

Collections of these VHH displaying phage are called a library. There are two kind of libraries which are commonly used. The immune library which consists of phage displaying VHHs derived from llamas or camels that were immunized with a certain antigen or pool of antigens (Van der Linden, 2000). The second library often used is the naïve library which consists of phage displaying VHHs isolated from a non-immunized, naïve, llama or camel (de Haard, 1999). A small amount of blood from these animals is used to isolate RNA, which is used as a template for reverse transciptase PCR to generate cDNA (see figure 4). From this cDNA the genes encoding the VHHs are amplified with specific primers. After restriction of the DNA encoding the VHH, this DNA can be cloned in a phagemid vector. Bacteria are transformed with these phagemid vectors containing the VHHs fused to the minor capsid protein gene III of the phage. The bacteria are used to produce phage displaying a wide variety of VHHs, normally 10^7 or more, all coupled to their respective DNA (see figure 4). One of the methods to select phage displaying the desired VHH from a large library is called biopanning (see figure 5). The VHHs displayed on the phages are allowed to bind to the antigen, which is attached to a solid surface. The antigen can also be captured to the solid surface by an (conventional) antibody binding the antigen or a tag on the antigen.

The phage with a VHH specific for the antigen binds and the remaining phage are thoroughly washed away. The binding phage can be eluted from the antigen, either with a pH shock or by specific elution with a competitor. This results in a pool of phage enriched for VHHs binding specifically to the antigen.

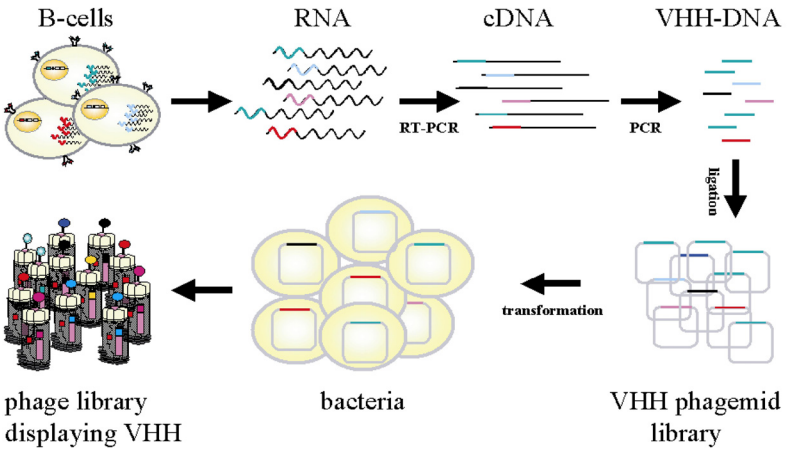

Figure 4 Schematic representation of the construction of a phage library.

These phage can be used to reinfect bacteria, which allows propagation of the DNA encoding the selected VHH and analysis of monoclonal VHHs. Consecutive rounds of biopanning can be performed to further enrich the phage pool. The amplification of the DNA by PCR resulting in a large, highly diverse pool of VHHs, combined with the easy selection via biopanning is another strength of phage display. One round of selection can be enough to select specific binders to a wide range of antigens out of a pool of 10^7 different VHHs.

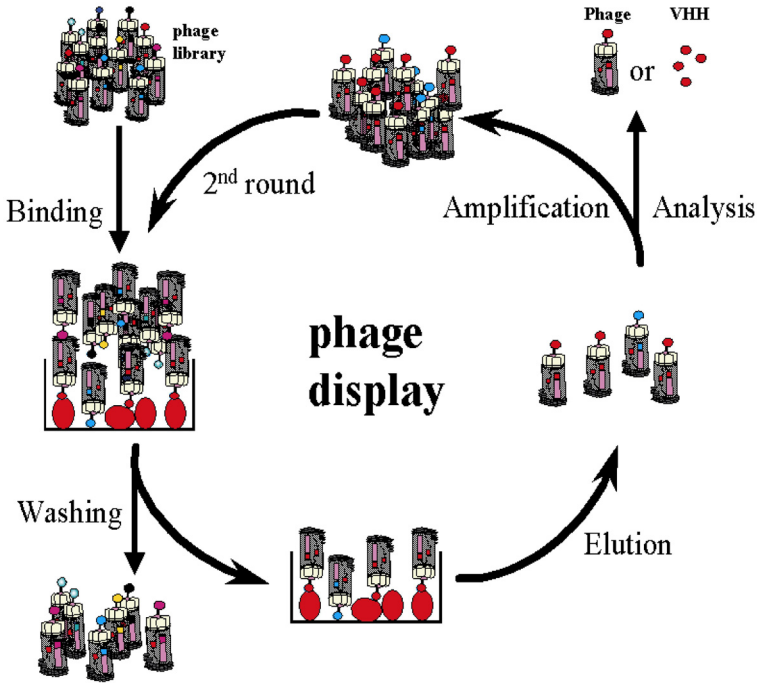

Figure 5 Schematic representation of biopanning.

A screening of the selected VHHs should be performed following biopanning, to investigate whether the selected VHHs meet the criteria set for the application in which they should be used. Usually, a simple high throughput ELISA binding assay is used for the primary screening of the VHHs for specific binding to the antigen. This primary screening has to be followed by a screening adapted to the application in mind.

The criteria which should be met by the VHHs are different for every application. High production levels and easy purification for cheap production, high stability in a wide range of non-physiological conditions, and specific, high affinity binding are just a few of these criteria. Together with the increasing number of applications in which antibody fragments play a key role this demands high flexibility in the method of selection of these antibodies.

Applied phage display allows for this flexibility. The only essential difference from the normal phage display procedure is that binding of the VHHs occurs in the presence of the application condition. Phage display performed in the presence of high concentrations of shampoo was shown to select for VHHs binding specifically to the antigen in shampoo (Dolk, in preparation). This could be a useful tool for circumventing laborious protein engineering after selection, when functionality under harsh conditions is required.

Outline of this thesis

The work presented in this thesis focuses on the possibilities of using llama heavy chain antibody fragments under extreme conditions. The high stability of VHHs make them unique binding blocks with a large potential for use in various applications.

The ability of reversible folding is a rather unique property of VHHs. Chapter two elaborates on the mechanism behind this reversible folding. We describe that VHHs which are unfolded at 80°C, are still able to form an antibody-antigen complex. The complex formation at 80°C implies that binding and refolding must have taken place at 80°C, induced by the antigen.

Another harsh condition for proteins are high concentrations of non- and anionic surfactants present shampoos. VHHs against *Malassezia furfur* were selected in an attempt to inhibit dandruff formation. The application of these VHHs requires storage in high concentrations of shampoo and functionality in diluted shampoo. Chapter three describes

the attempts to select VHHs functional in shampoo. Phage display performed in the presence of shampoo resulted in VHHs binding in shampoo. The higher stability of these VHHs was further investigated on structural changes resulting in this higher stability.

The use of antibodies for the inhibition of viruses is not a new concept. However, cost effective antibody treatment to prevent viral infection with conventional antibodies is not feasible. Reports have shown that low pH and digestion of antibodies by proteases significantly decrease the functionality of orally administered antibodies (Petschow, 1994). VHHs inhibiting rotavirus have been selected at low pH and in the presence of pepsin (Van der Vaart, in preparation). Chapter four describes the improvement of one good candidate VHH by protein engineering. This resulted in a mutant with decreased trypsin susceptibility, higher thermostability, higher production levels and equal affinity, which could be an economically feasible product for the prevention of rotavirus in developing countries.

Chapter five reviews the three mutational studies performed in chapter two, three and four. Surprisingly, these three unrelated studies revealed that mutations in the "former" VH-VL interface of the VHH, resulted in an effect on stability. Chapter five describes the differences between residues in VHs and VHHs in this area, and speculates on an important role for these residues in stability.

Chapter six describes the results of this thesis in a broader perspective. Emphasis is on the stability of VHHs and their adaptation to the single domain status.

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Chapter 2

Induced refolding of a temperature denatured llama heavy chain antibody fragment by its antigen

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Abstract

In a previous study we have shown that llama VHH antibody fragments are able to bind their antigen after a heat shock of 90°C, in contrast to the murine monoclonal antibodies. However, the molecular mechanism by which antibody:antigen interaction occurs under these extreme conditions remains unclear. To examine in more detail the structural and kinetic aspects of the binding mechanism, an extensive CD, ITC and NMR study was initiated. In this study the interaction between the llama VHH -R2 fragment and its antigen, the dye Reactive Red-6 (RR6) has been explored. The data show clearly that most of the VHH-R2 population at 80 °C is in an unfolded conformation. In contrast, CD spectra representing the complex between VHH-R2 and the dye remained the same up to 80 °C. Interestingly, addition of the dye to the denatured VHH-R2 at 80 °C yielded the spectrum of the native complex. These results suggest an induced refolding of denatured VHH-R2 by its antigen under these extreme conditions. This induced refolding showed some similarities with the well established 'induced fit' mechanism of antibody-antigen interactions at ambient temperature. However, the main difference with the 'induced fit' mechanism is that at the start of the addition of the antigen most of the VHH molecules are in an unfolded conformation. The refolding capability under these extreme conditions and the stable complex formation make VHHs useful in a wide variety of applications.

Introduction

Antibody-antigen interaction has been well described in recent years. On complex formation, conformational changes of antibody and/or antigen can occur. Koshland , first described conformational changes that induced an active state in an enzyme, subtilisin, by a substrate molecule; this mechanism was called 'induced fit' (Neet and Koshland 1966). This mechanism can be described as fast bimolecular association followed by a unimolecular isomerisation. Meanwhile, examples of this 'induced fit' mechanism have been described for both antibody-antigen interaction as well as other protein-protein interactions and protein-DNA interactions (Webster, 1994; McEwan, 1996; Burley, 1994).

The equilibrium between the complex and the uncomplexed antigen and antibody fragment is described by a simple model, the law of mass action. The equilibrium will set when the rate at which new antibody-antigen complexes are formed equals the rate at which the antibody-antigen complexes dissociate. However, whether the equilibrium is directed towards the complex or the dissociated antibody and antigen depends on a lot of factors, like the temperature. Extreme conditions necessary for a lot of applications in research or the industry can shift this equilibrium in such a way that the antibodies are not useful any more.

A novel class of IgG antibodies was discovered in camelids by Hamers-Casterman and co-workers, (1993). These antibodies are devoid of light chains and are referred to as heavy chain antibodies. The binding part of the heavy chain antibodies comprises only a single domain, called VHH. Camelid VHH's display similar functional characteristics with respect to specificity and affinity as compared to classical antibodies (Van der Linden, 1999; Muyldermans, 2001). We have shown that the llama VHH antibody fragments can be produced at high levels in *Saccharomyces cerevisiae,* up to 2.5 mg l^{-1} OD_{660}^{-1} in shake flask or ten-fold higher with fed-batch fermentation at 10 L scale (Frenken, 1998; Frenken, 2000; Thomassen, 2002). A remarkable difference between the llama antibody fragments and the convential antibody fragments is the apparently higher heat stability of the llama VHH's (Van der Linden, 1999). Van der Linden showed that some of the VHH fragments were able to bind their antigen even at a temperature as high as 90 °C. Other fragments were found not able to bind their antigens at this temperature, but could bind their antigen upon cooling down. We have shown that one of these llama VHH antibody fragments against the human pregnancy hormone hCG is indeed able to refold into its native conformation after exposure to high temperatures (Pérez, 2001), in contrast to conventional antibodies which tend to aggregate. This results in a higher intrinsic stability of VHHs (Ewert, 2002; Conrath, 2003). The question arose whether the VHH antibody fragments which can bind their antigen at high temperatures still have their native structure or whether refolding of the antibody takes place induced by addition of the antigen.

In order to obtain more insight into the mechanism of binding at these extreme temperatures, we have used the VHH fragment R2, which binds specifically to the copper containing azo-dye RR6 (Van der Linden, 2000). This chemical antigen, the azo-dye RR6, has the advantage that it is not altered by the extreme conditions used in these experiments.

CD spectroscopy was used to determine if the VHH-R2 fragment was completely unfolded at high temperatures and whether addition of the antigen at high temperatures could induce complex formation of the VHH-R2 fragment and its antigen. Other antibody fragments and different antigens were used to determine if this mechanism was generic or specific for VHH-R2. NMR spectroscopy was used to obtain more detailed information on the structure of VHH-R2 during unfolding, binding and refolding. Isothermal titration calorimetry was used to investigate the kinetic aspects of the interaction between VHH-R2 and the dye.

We have found that the majority of the population of VHH-R2 molecules is unfolded at 80 °C. However, the preformed complex of VHH-R2 and the dye is not affected by the increase in temperature. Surprisingly, addition of the antigen at 80 °C was able to refold VHH-R2 to its native conformation.

The remarkable refolding properties of VHH domains and the stability of the complex make them very suitable for various applications. For instance, we have shown that they can be used over 2000 times in affinity purification without any measurable loss of binding capability (Verheesen, 2003). But also for applications in protein arrays they proved to be very suitable (Zhu, 2000). On one hand they can make very stable complexes with their antigen with binding properties exceeding traditional antibodies, on the other hand the chemically modification necessary to link them on a support does not alter their binding properties.

Material and methods

Fragments used in this study

The fragments used in this study are the binding domains of Llama heavy chain antibodies (VHH). VHH-R2 against the azo-dye RR6, VHH-A52 against the azo-dye RR1, and VHH-H14 against the human pregnancy hormone hCG were used in this study. The isolation of VHH's against the azo-dye RR6, the azo-dye RR120 (dimer of RR1) and the human pregnancy hormone (hCG) has been described elsewhere (Spinelli, 1996; Frenken, 2000). The VHH's were produced in *Saccharomyces cerevisiae* via batch fermentation (Van der Linden, 2000). VHH's were purified using a 5 ml ProteinA column (Hi-Trap, Pharmacia). Uniformly ^{15}N-labelled VHH-R2 was produced by using ^{15}N-labelled ammonium sulphate as the sole nitrogen source. The affinities of the antibodies were in the

nanomolar range (Van der Linden, 1999). VHH-R2, k_{on} = 1.54 x 10^5 M^{-1} s^{-1}; k_{off} = 3.34 x 10^{-3} s^{-1}; K_D = 22.0 nm.

Circular dichroism

CD spectra were collected using a thermostated (20-80 ˚C) Jobin-Yvon CD6 Dichrograph Circular Dichroïsm spectrophotometer. The data were processed with the Dichrograph software version 1.3. Experiments in the near ultra violet region (250-350 nm) were performed with 37 μM antibody fragment in PBS pH 7.4 in 10 mm cylindrical quartz cuvettes (Helma). The reactive chloride groups of the azo-dye RR6 were blocked in 1M NH_4Cl. After blocking, the RR6 was transferred to PBS buffer pH 7.4. The spectral bandwidth was automatically kept at 2 nm. The wavelength increment was 0.2 nm/step and the accumulation time was 1 s/ step. Each spectrum resulted from averaging five successive scans. The spectra of the solvent alone and solvent with RR6 or RR1 were recorded under identical conditions and were subtracted from the protein spectra.

Experiments in the far ultra violet region (190-250 nm) were performed with 90 μM peptide in ddH2O in 2 mm quartz cuvettes (Helma). The reactive chloride groups of the azo-dye RR6 were blocked in 1M NH_4Cl. After blocking, the RR6 was transferred to PBS buffer pH 7.4. Each spectrum resulted from averaging five successive scans. The spectra of the solvent alone and solvent with RR6 were recorded under identical conditions and were subtracted from the peptide spectra.

NMR

For the NMR measurements, uniformly ^{15}N-labelled VHH-R2 samples were prepared in a buffer containing 10 mM deuterated sodium acetate (99.5% D) pH 4.6, and 100 mM NaCl. The protein concentration was 0.1 mM and the samples contained 90% H_2O and 10% D_2O (99.9% D). All NMR data sets were collected on a Bruker 600 MHz DMX spectrometer equipped with a 5 mm inverse triple-resonance probehead (1H/15N/13C) with a self-shielded z-gradient coil and a variable temperature unit. [^{15}N,^{1}H] water flip-back HSQC (HSQC) spectra were measured (Mori, 1995). The spectral width and number of complex points in the ^{15}N dimension was 1672 Hz (folding-in some backbone resonances) and 128 respectively and 9259 Hz and 2048 in the ^{1}H dimension. Quadrature detection in

the indirectly detected dimensions was accomplished using the States-TPPI acquisition method (Marion, 1989). NMR data were processed using XWINNMR software from Bruker. All dimensions were apodised using shifted sine-bell windows and zero-filled to the next power of two to improve the digital resolution.

Isothermal titration calorimetry

ITC was performed using a Microcal Omega titration microcalorimeter (Microcal, Inc.) connected to an external waterbath. All measurements were performed using samples which were degassed just before use. To investigate the binding of RR6 to VHH-R2 experiments were performed with 33 μM antibody fragment in PBS pH 7.4 in the cell and 0.875 mM blocked RR6 in PBS buffer pH 7.4 in the syringe. Cell volume was 1,345 ml of VHH-R2. Fifty injections of 6μl of RR6 were performed. The solution was stirred at 700 rpm. In all cases, injections were carried out over a 15 s period with a 4 min equilibration period between each injection, which was sufficient for the baseline to be re-established. Data analysis was performed using the ITC data analysis software Microcal origin (Microcal, Inc.).

Results and Discussion

CD spectroscopy

In order to collect information on the tertiary structure of the VHH antibody fragments, CD spectra were taken in the near UV area (250-350 nm) at different temperatures. Figure 1a shows the CD spectrum of VHH-R2 at 20 °C, which is considered to be the native state. The clear negative peaks shown in this spectrum at 281, 290 and 297 nm represent the CD bands of the aromatic residues present in the VHH-R2 molecule (line 1), in particular the stacking interactions of the chromophor side chains of the tryptophane molecules. Subsequent incubation of the VHH-R2 sample at 70 °C for 120 minutes does not affect the CD spectrum (data not shown), indicating that the structure of the fragment remains unchanged. However, incubating the VHH-R2 at 80 °C for 10

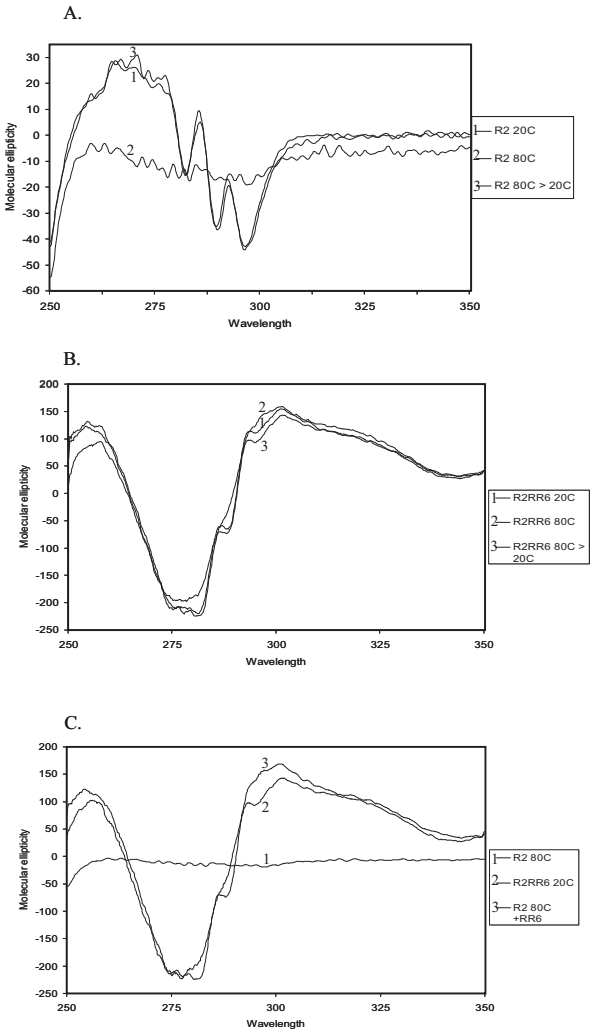


Figure 1 A) CD spectra in the near UV area (250 –350 nm) of VHH-R2 at 20 °C (line 1), 80 °C (line 2) and after cooling down to 20 °C (line 3). B) CD spectra in the near UV area (250 –350 nm) of the VHH-R2:RR6 complex at 20 °C (line 1), 80 °C (line 2) and after cooling down to 20 °C (line 3). C) CD spectra in the near UV area (250 –350 nm) of VHH-R2 at 80 °C (line 1), of the VHH-R2:RR6 complex at 20 °C (line 2) and of VHH-R2 at 80 °C after addition of a 1:1 molar ratio of preheated RR6 (line 3).

minutes dramatically alters the CD spectrum of VHH-R2, resulting in the disappearance of the CD bands (line 2).

The hydrophobic stacking interactions between the chromophor side chains are disturbed, indicating an unfolded conformation of the fragment. Subsequent cooling of the VHH-R2 sample back to 20 °C rendered the native spectrum (line 3). This indicates that heat induced unfolding of VHH-R2 is reversible, comparable to VHH-H14 [11]. Figure 1b shows the CD spectra of the VHH R2-RR6 complex (line 1). The complex was formed by addition of the dye RR6 to a 1:1 molar ratio at 20 °C. The CD spectrum of VHH-R2 with RR6 is different from the CD spectrum of VHH-R2 alone.

The strong CD bands are probably due to stacking interactions of the aromatic side chains, i.e.Trp52A in CDR2 of VHH-R2 is stacked against the naphtyl moiety of the azo-dye RR6 as shown in the X-ray–structure of the complex [18]. No signals have been observed in the CD spectrum of the hapten dye RR6 in PBS. Heating the VHH-R2-RR6 complex to 80 °C did not change the CD spectra (line 2), even after incubation for 1 hour at 80 °C. Subsequent cooling down of the complex does not alter the spectrum (line 3). This indicates that the complex retains its native conformation at this temperature. The complex of VHH-R2 with its antigen RR6 is more stable than VHH-R2 alone at high temperatures.

To obtain more insight into the stability of the complex at high temperatures, the VHH-R2 was heated to 80 °C and after equilibration for 5 minutes, the antigen RR6 was added to the unfolded VHH-R2. Figure 1c shows the CD spectrum of the unfolded VHH-R2 at 80 °C (line 1). Subsequent addition of a 1:1 molar ratio of preheated RR6 to the antibody at 80 °C resulted in the CD spectrum shown in figure 1c (line 3). Surprisingly, this spectrum shows all the characteristics of the spectrum of the antibody-antigen complex formed at 20 °C (line 2). This indicates that at 80 °C the VHH-R2-RR6 complex adopts the same conformation as the complex formed at 20 °C.

Control experiments were performed regarding antigen specificity, antibody specificity and the effect of the copper in the dye on the CD spectra. To investigate whether the refolding was antigen specific, experiments were performed with VHH-R2 and the azo-dye RR1. The molecular structure of RR1 is different from RR6 and it does not contain copper like RR6 (figure 2).

ELISA experiments at room temperature showed crossreactivity of VHH-R2 with RR1 (data not shown). The CD spectrum of VHH-R2 after addition of the dye RR1 was different from the CD spectrum of VHH-R2 alone and also different from the CD spectrum of the VHH-R2 – RR6 complex.

This indicates that VHH-R2 is able to form a complex with RR1. However, addition of RR1 to the unfolded VHH-R2 at 80 °C does not lead to the native complex of R2:RR1 (data not shown).

To ensure that the refolding of the VHH-R2 was not due to an aspecific interaction with RR6, the RR6 azo-dye was added to a different llama VHH fragment, VHH -H14 raised against hCG.

Figure 3a shows the CD spectrum of H14 alone at 20 °C (line 1). Addition of RR6 did not alter the spectrum, indicating that the VHH–H14 does not interact with RR6 (line 2). Accordingly, at 80 °C the VHH-H14 is completely unfolded (line 3) and addition of the RR6 at 80 °C did not alter the spectrum as well (line 4). To exclude that the copper alone has an effect on the CD spectrum of VHH-R2, CD experiments were performed with VHH-R2 in the presence of $CuCl_2$ and $CuSO_4$. Addition of $CuCl_2$ or $CuSO_4$ to VHH-R2 at 20 °C did not change the spectra of VHH-R2 This indicates that the copper is not responsible for the large bands in the CD spectrum of VHH-R2 (data not shown).

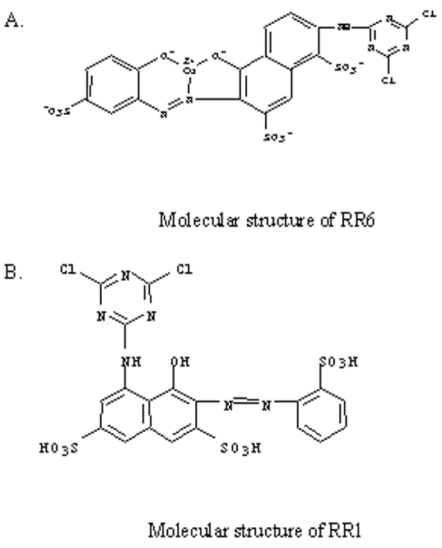


Figure 2. Structure of the copper containing azo-dye RR6 (A) and the azo dye RR1 (B).

A different llama antibody fragment, VHH-A52 raised against the azo dye RR1, was used to determine whether the refolding at high temperatures was specific for VHH-R2 and RR6 or if it is a more generic process. The same set of experiments as with VHH-R2 was performed. Figure 3b shows the spectra of VHH-A52 (line 1) and the complex of VHH-A52:RR1 at 20 °C (line 3). Heating the VHH-A52 to 80 °C showed that the majority of the VHH -A52 pool is in an unfolded conformation (line 2). Addition of RR1 to the unfolded VHH-A52 at 80 °C led to complex formation and rendered a spectrum identical to the native complex spectrum at 20 °C (line 4). This clearly indicates that VHH-A52 is capable of refolding upon addition of its antigen at 80 °C just like VHH-R2 with its antigen RR6. Addition of RR6 at 20 °C and 80 °C to VHH-A52 also did not alter the spectra of VHH-A52 (data not shown). These observations indicate that refolding at 80 °C upon addition of the antigen was not specific for VHH-R2, but could be generic for the llama antibody fragments.

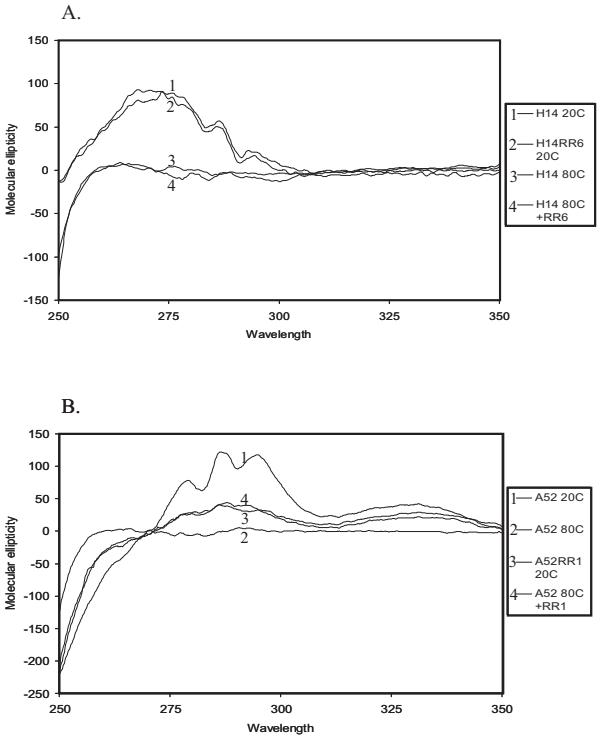


Figure 3 A) CD spectra in the near UV area (250 –350 nm) of VHH-H14 at 20 °C (line 1), with RR6 at 20 °C (line 2), at 80 °C (line 3) and with RR6 at 80 °C (line 4). B) CD spectra in the near UV area (250 – 350 nm) of VHH-A52 at 20 °C (line 1), at 80 °C (line 2), of the VHH-A52:RR1 complex at 20 °C (line 3) and of VHH-A52 at 80 °C after addition of a 1:1 molar ratio of preheated RR1 (line 4).

Peptide binding

In order to investigate whether an interaction between denatured VHH-R2 and RR6 is possible, a peptide was synthesized comprising the CDR3 binding loop of VHH-R2. Figure 4 shows the CD spectrum of the peptide (line1). The spectrum of the peptide

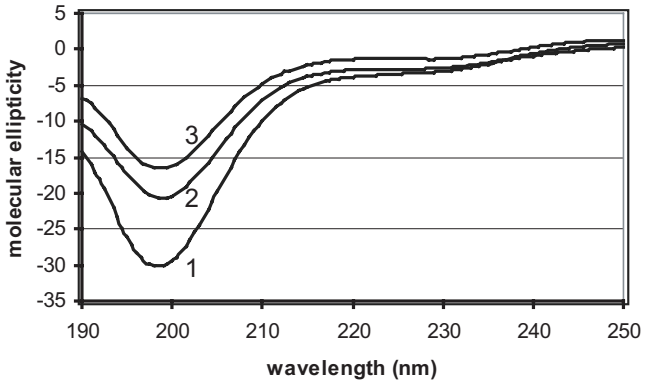
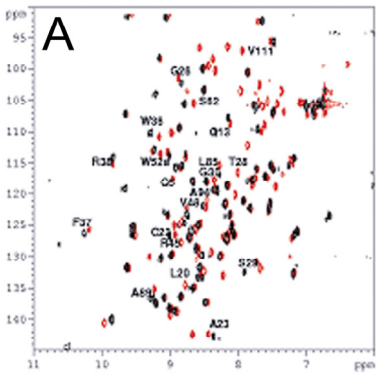


Figure 4 CD spectra in the far UV area (190-250nm) of the CDR3 peptide without RR6 (line 1), after addition of a 1:1 molar ratio of RR6 (line 2), and after addition of a 1:3 molar ratio of RR6 (line 3).

indicates that the CDR3 peptide is largely in an unordered random coil formation. Addition of antigen RR6 to the peptide renders a spectrum as shown in figure 4. This shows that there is an interaction between RR6 and the peptide. The spectra with dye shows a shift in the 190-210 nm range, which indicates that the antigen RR6 induced a more structured conformation of the peptide. All spectra shown are corrected for the spectrum of the buffer or buffer with the corresponding concentration of RR6. Therefore the shift of the curve can not be an effect of pH. These results indicate that the antigen RR6 is able to interact with the unordered CDR3 peptide.

NMR

To obtain more details on the structure of VHH-R2 upon binding of the dye RR6, NMR experiments were performed. HSQC spectra provide information on the chemical shift of the amide resonances in the backbone of R2. A well-dispersed amide cross-peak pattern is representative for a native protein. Figure 5a shows the superimposed HSQC spectra of free ^{15}N-VHH-R2 (red) and the ^{15}N-VHH-R2:RR6 complex (black) at 27 °C. Both spectra clearly show that ^{15}N-VHH-R2 and its complex sample is folded. Moreover, a few cross-peaks in the spectrum of the ^{15}N-VHH-R2:RR6 complex were shifted and some of them were even undetectable. This indicates that these resonances probably correspond to the residues in close proximity to RR6 or to residues which undergo long range conformational changes upon binding of RR6. The reason that some of the cross-peaks are undetectable is due to line-broadening induced by the interaction of the resonating nuclei with the unpaired electron of the paramagnetic copper center in RR6. The relaxation of the protons in close proximity to the paramagnetic centeris fast and leads to moderately/largely broadened signals beyond detection limit. The chemical shift assignments for most of the perturbed residues have been obtained and are assigned in figure 5a (the assignments were kindly provided by Herve Darbon). The broadened cross-peaks in the spectrum are L20, T28, S29, G30, R45, W52a, S62, A94, and V111. In the X-ray structure W52a is in stacking contact with the naphtyl moiety of RR6 and is therefore undetectable in the HSQC experiment of the complex (Spinelli, 2000). Unfortunately, the NMR resonance assignments of the coordinating ligand of the copper, H31a and H31c, are still missing. A few other residues in CDR1 close to the histidines co-ordinating the copper i.e. T28, S29



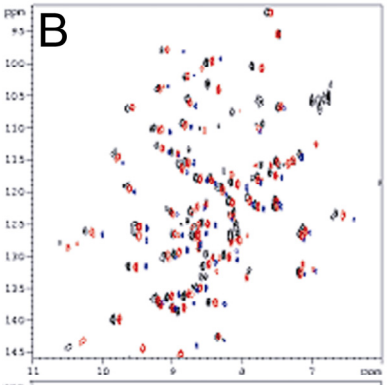
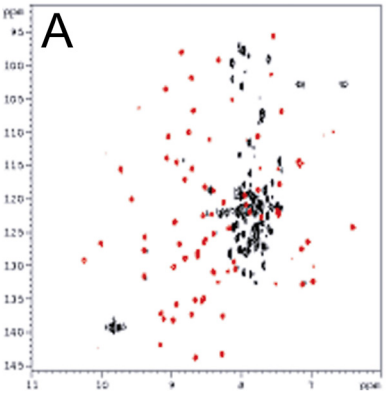


Figure 5 A) Superposition of the HSQC experiments of 0.1 mM VHH-R2 (red) and VHH-R2:RR6 complex (black) with the assignments of the shifted and broadened amide resonances. B) Superposition of the HSQC experiments of the VHH-R2:RR6 complex at three different temperatures: 27 °C (black), 50 °C (red) and 80 °C (blue).



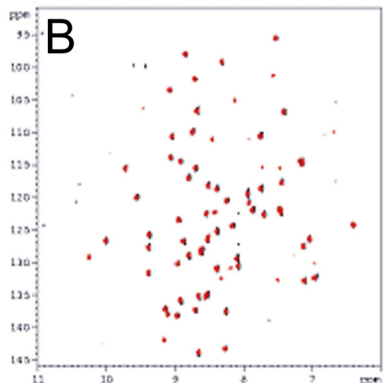


Figure 6 A) Superposition of the HSQC experiments of 0.1 mM VHH-R2 (black) at 80 °C and after the addition of 0.1 mM RR6 at the same temperature (red). B) HSQC spectrum of the VHH-R2:RR6 complex heated to 80 °C (black) superimposed with the spectrum of the complex prepared at 80 °C (red).

and G30 were also severely broadened in the complex. The rest of the undetectable residues with the exception of L20 were either part of or are close to the CDR's. The ^{15}N-VHH-R2:RR6 complex was heated from 27 °C to 80 °C (Fig. 5b). The dispersion of the amide resonances remained roughly the same up to 80°C except for a small shift of the peaks not representative of large conformational changes.

However, the intensity of the cross-peaks dropped substantially at higher temperatures due to line broadening which could be indicative of aggregation. In contrast, when free ^{15}N-VHH-R2 is heated to 80 °C a large shift of the amide resonances to random coil chemical shift values has been observed (Fig. 6a).

This suggests that the majority of the population of the ^{15}N-VHH-R2 molecules is unfolded at 80 °C. Addition of an equivalent molar amount of RR6 to denatured ^{15}N-VHH-R2 at 80 °C rendered a dramatically altered spectrum (Fig. 6a). Remarkably, this spectrum appeared to be identical to that from the ^{15}N-VHH-R2:RR6 complex prepared at 27 °C and subsequently heated to 80 °C (Fig. 6b).

This finding gives direct evidence for the refolding of non-native ^{15}N-VHH-R2 at 80 °C upon binding of its antigen RR6. The NMR experiments were performed at pH 4.6 to minimise the exchange of amide protons. Deviations because of differences in pH between the CD and NMR experiments were checked by repeating the CD experiments at pH 4.6, no differences were observed. The NMR data confirms the CD data and gives direct evidence for the hypothesis of ‘induced refolding’.

Isothermal Titration Calorimetry

To understand the kinetics involved in the refolding process isothermal titration calorimetry (ITC) was performed. Small amounts of RR6 were titrated to VHH-R2 at 20 °C and 80 °C. Figure 7a shows a typical binding isotherm of RR6 to VHH-R2 at 20 °C.

The measured heat change for each injection is negative up to a molar ratio of 3 , after which it remains constant up to 7 equivalents of RR6. The exothermic signals of –8 kcal/mol at molar ratios up to 3 represent the heat change of complex formation of RR6 to VHH-R2. The enthalpic signals at molar ratios between 3 and 7 represent the heat of dilution of the RR6 and are observed in a control titration, wherein RR6 was titrated to PBS buffer only. Figure 7b shows the binding isotherm at 80 °C. The measured heat change for

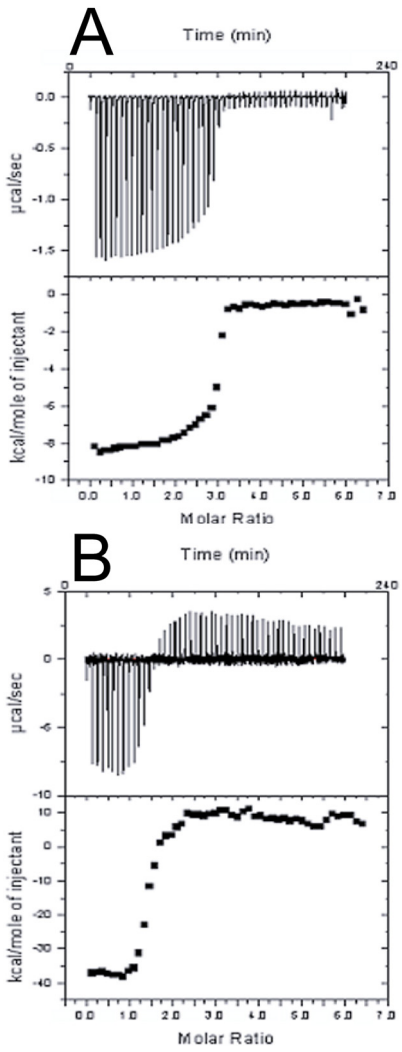


Figure 7 A) Binding isotherm of the titration of RR6 to VHH-R2 at 20 °C. Top panels are the raw ITC data. Lower panels are the integrated calorimetric heats. B) Binding isotherm of the titration of RR6 to VHH-R2 at 80 °C. Top panels are the raw ITC data. Lower panels are the integrated calorimetric heats.

each injection is negative up to a molar ratio of 1.5 , after which it remains constant up to 7.0 equivalents of RR6. The exothermic signals of –39 kcal/mol represent the complex formation of RR6 to VHH-R2 and the refolding of VHH-R2.Values for the enthalpy change and the number of binding sites (N) correspond to the intercepts and midpoints, respectively, of the titration curves and can be obtained directly from the ITC data. Both curves were fitted with a model assuming two binding events, because two RR6 molecules were shown to bind to VHH-R2 (Spinelli, 2000) (see table 1).

The binding isotherms of the titration of RR6 to VHH-R2 at 20 °C and 80 °C shows that titration of RR6 is exothermic at both temperatures. The observed enthalpy change indicates that refolding takes place at 80 °C and confirms the CD data. At 20°C the kinetic parameters of the binding of the first and the second RR6 molecule are almost identical. At 80°C the kinetic parameters of the binding of the first RR6 molecule are comparable to the parameters at 20°C, although the association constant is slightly lower. However, the kinetic parameters of association of the second RR6 molecule are drastically different, suggesting that at 80°C the second RR6 molecule is not able to bind efficiently. The data obtained from the crystal structure of the complex show two binding sites for

	N1	Ka 1 (M^{-1})	ΔH1 (J/mol)	ΔS1 (J/mol/K)	N2	Ka 2 (M^{-1})	ΔH2 (J/mol)	ΔS2 (J/mol/K)
RR6 to R2 at 20°C	1.4	$3.4x10^6$	-7400	4.6	1.6	$4.6x10^7$	-8446	6.3
RR6 to R2 at 80°C	1.4	$1.9x10^6$	$-3.9x10^4$	-83	6.1*	350.2*	$1.7x10^{6}$ *	503*

Table 1. The calorimetric data corresponding to the RR6 titrations at 20°C and 80°C fitted using a model assuming two binding events. The asterix indicates that the accuracy of these results are very poor.

RR6, however the RR6 monomer was the authentic hapten and our model is based on binding of a monomer RR6 by VHH-R2. The large enthalpic change of -35 kcal/mol at 80 °C can be explained because not only binding and complex formation, like at 20 °C, but also refolding occurs. The ITC data confirm the CD data and show that the kinetic parameters of binding at 80°C is remarkably comparable to binding at 20°C.

Conclusions

We have shown by CD spectroscopy and NMR that unfolded VHH-R2 was able to form a native complex after addition of its antigen RR6 at high temperature, indicative of refolding of VHH-R2 at 80 °C. Figure 8 shows a scheme of the possible steps in the binding process of a VHH to its antigen at 80 °C. A possible explanation for complex formation at these high temperatures is that the small fraction of native VHH-R2 at 80°C is able to form a complex with the dye RR6.

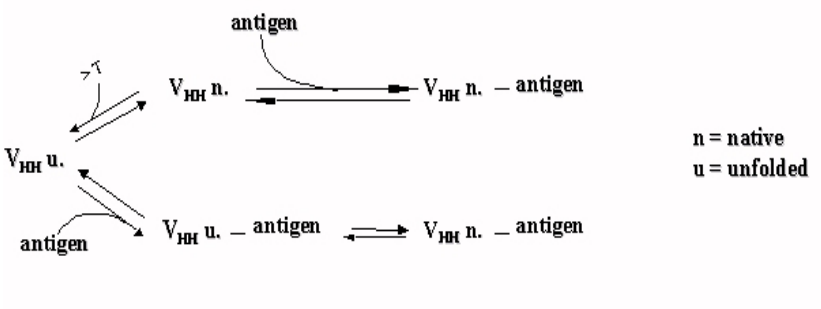


Figure 8. A reaction scheme of native and unfolded VHH and its antigen towards complex formation with two suggestions for the role of addition of antigen in induced refolding at high temperature.

Consequently, the equilibrium will set, according to the law of mass action, leading to a new equilibrium with the majority of the VHH-R2 in complex with RR6. Another explanation for complex formation could be that the antigen first binds to unfolded VHH-R2, which subsequently induces the refolding process. Figure 4 in this paper shows that an interaction between the unordered CDR3 peptide and the dye RR6 is possible. This can be explained in more detail after careful inspection of the X-ray structure. The X-ray data showed a dimer of RR6 bound to the VHH , however only one RR6 molecule of the dimer is buried in a cavity at the surface of the VHH (Spinelli, 2000). This first half of the dimer is buried for 60% in the VHH , embedded between the three CDRs. Binding of a RR6 molecule to CDR3 increases the probability that the RR6 molecule binds to a second CDR, resulting in formation of the cavity as present in the native VHH-R2.

RR6 was shown to induce structural changes in the unstructured CDR3 peptide. These structural changes in unfolded VHH-R2 could increase the probability of reformation of the loops of the CDRs. This could be the folding nuclei that allows long range interacting residues to come in more proximity of each other and refold to the correct native conformation, thereby trapping the unfolded VHH-R2 into a stable conformation, the VHH-R2:RR6 complex . In this case the antigen RR6 would act like a small chaperone molecule, which acts by increasing the probability of unfolded proteins to form a stable structure (Hendrick and Hartl, 1995; Ellis and Hartl, 1999; Torok, 2001). However, the difference is that small chaperone molecules often bind to regions that are buried in the native protein, but exposed in the unfolded protein, whereas RR6 binds to the CDRs that are also exposed in the folded VHHs. The main difference between these two pathways is whether RR6 binds to the unfolded VHH-R2 or to the native VHH-R2 at 80 °C. In this perspective, it was interesting to see that the azo-dye RR1 did crossreact with native VHH-R2 to some extent at 20 °C. However, addition of RR1 to the unfolded VHH-R2 at 80 °C did not induce refolding of VHH-R2. The interaction of RR1 with VHH-R2 is probably not sufficient to induce refolding. Comparing the molecular structures of RR1 and RR6, there are some remarkable resemblances, the aromatic rings, the three sulphonate groups and the sulfonyl-phenol group. Inspection of the contacts of VHH-R2 with RR6 shows that CDR1 is in contact with RR6 via the copper molecule, CDR2 is in contact with the three sulphonate groups of RR6 and CDR3 with the p-sulfonyl-phenol group of RR6 (figure 9). RR1 does not contain a copper, the sulphonate groups of RR1 are differently orientated compared

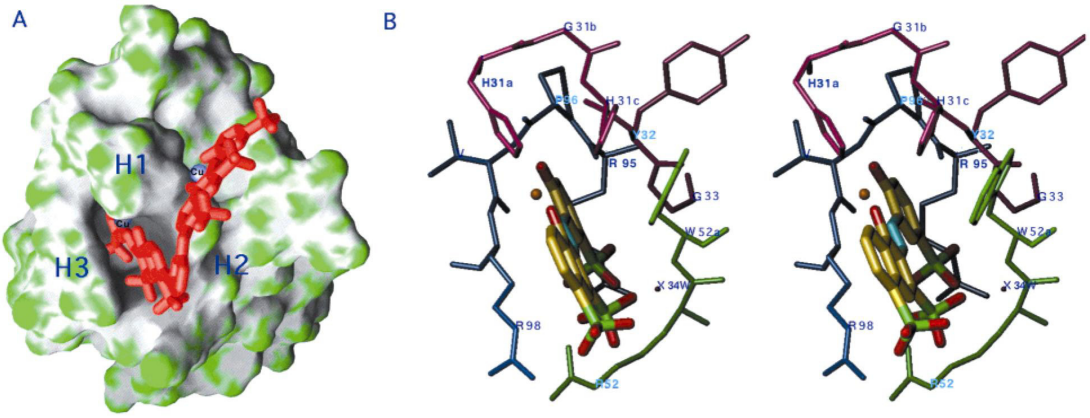


Figure 9. The combining site of the anti-RR6-VHH and the RR6 inside, viewed from the top. A) Water-accessible surface of the VHH displaying the cavities, with the bound RR6 molecule (red) in ball-and-stick representation, with the copper ions as silvery blue spheres. The green colour identifies the protruding surfaces, and the gray colour identifies the reentring surfaces (GRASP representation, 32). The localisation of the three CDRs has been indicated by H1, H2, and H3. B) Stereoview of the P1 and N1 groups of the P1+N1 groups of the RR6 molecule in complex with the 3 CDRs of the anti-RR6-VHH fragment. The CDRs 1-3 are red, green, and blue, respectively. (Reproduced with permission from Biochem. 2000, 39(6), 1217-1222. Copyright 2000 Am. Chem. Soc.)

to RR6, and thus it is likely that the contact of RR1 to VHH-R2 is with the identical p-sulfonyl-phenol group of RR1 to CDR3 of VHH-R2. The reason why RR1 is not able to induce refolding by long range interactions could be that stabilisation of the CDR loop(s) followed by refolding is not efficient with the antigen RR1.

VHH-A52, a VHH selected against and binding specifically to the azo dye RR1, did show refolding after addition of RR1 at 80 °C. In this case the interaction of RR1 with VHH-A52 is sufficient to induce refolding. RR1 is not only able to bind to VHH-A52, but is also capable of inducing long range interactions in VHH-A52 necessary to refold VHH-A52 to its correct native conformation. Spinelli reported the structure of VHH-A52 with RR1 (Spinelli, 2001). Where VHH-R2 forms a cavity with its three CDRs and binds RR6 in a central combining site, VHH-A52 binds RR1 on a lateral combining site with CDR2,

CDR3 and a framework residue. Thus, although the mechanism for binding their antigen is different between VHH-A52 and VHH-R2, both VHHs are able to refold upon addition of their antigen at 80 °C. We concluded that refolding at 80 °C upon addition of the antigen is not specific for the VHH-R2, but is generic for the llama antibody fragments against organic haptens. However, high affinity binding of VHH to its antigen is a necessity to enable long range interactions to occur.

The binding of camelid single domain antibody fragments under extreme conditions increases the possibilities to use these simple binding molecules in a wide range of applications. They are not only suitable for applications where a relatively short rise in temperature is necessary like with sterilization, but they may also be used in application at higher temperature or under different denaturating conditions. Because of the single domain 'induced refolding' is a unique characteristic of llama antibody fragments in general.

This paper shows that the increased stability reported in a lot of articles concerning single domain antibodies derived from camelids might be contributed for a large part by their reversible folding and capability of binding to their antigen under extreme conditions. This was shown by the fact that the unfolded VHH was still functional, that is: able to efficiently bind to their antigen to a great extend in a relatively short time span.

Acknowledgements

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Chapter 3

Applied phage display in harsh detergent conditions, implications for antibody stability

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Abstract

Phage display of llama single domain antibody fragments (VHHs) can select VHHs that function well under a wide range of application conditions. We show here that this technique can even be extended to very harsh conditions such as the presence high concentrations of shampoo. In the presence of shampoo, containing non- and anionic surfactants, we selected several VHHs that all bind the cell wall protein Malf1 of the dandruff causing *Malassezia furfur*. These VHHs show high stability in the presence of shampoo, but also under other denaturing conditions, like a high urea concentration. Many of the stable VHHs were found to have an arginine at position 44. Introduction of this arginine in the most stable VHH that lack this arginine resulted in a dramatic further of its stability. The combination of the unique properties of VHHs with applied phage display, and protein engineering, is a powerful method to obtain highly stable VHHs that can be used in a wide range of applications.

Introduction

The human scalp houses a very complex balance between many micro-organisms. One of these organisms, *Malassezia furfur* (*M. furfur*) is an anthropophilic fungus that belongs to the physiological skin flora. This fungus can grow in a yeast phase and in a mycelial phase. *M. furfur* has been implicated in the formation of dandruff (6, 12, 42, 46) and other skin diseases, like psoriasis (1). Compared to people without dandruff, significantly increased numbers of *M. furfur* were found on the scalp of those with dandruff (36, 43) Reduction of the number of *M. furfur* cells on persons suffering from dandruff resulted in a decrease in the severity of dandruff (34).

To date, the treatment and/or prevention of dandruff has involved the use of chemical anti-fungal compounds (26), compounds such as ketoconazole (35), selenium sulfide (6), ciclopirox olamine, piroctone-olamine, zinc pyrithione and sulfur-containing substances (42).

We here demonstrate a fully novel approach to inhibit *M. furfur* by inhibition with antibodies. For successful application of antibodies in consumer goods they must meet

certain requirements regarding cost of production, specificity, affinity and especially the stability of the antibody under application conditions.

Camelid heavy chain antibodies have been shown to be a useful tool in many biotechnological applications (10, 14, 27, 47). The variable domains of these heavy chain antibodies (VHH) form a single binding domain (15). The simple, one domain structure of these VHHs give them unique characteristics with respect to production, folding and stability (13, 33). Furthermore, camelid VHHs display similar behaviour with respect to specificity and affinity as compared to classical antibodies (45). The extra long protruding CDR3 of VHHs is implicated in the efficiency of inhibition of enzymes and small organisms (7, 8, 10, 22). Therefore VHHs are good candidates for antibody-mediated delivery of anti-dandruff agents or even direct neutralization of *M. furfur*.

Phage display is a common method for the selection of specific antibody fragments under laboratory conditions (18). We describe the selection of VHHs specific for the *M. furfur* cell surface protein (Malf1) via phage display under application conditions, which in this case are a high concentration of non- and anionic surfactants present in shampoos, like Andrelon™ and Organics™.

Surprisingly, phage display can select for VHHs binding specifically to Malf1, even under harsh application conditions such as high concentrations of shampoo. In addition, it was shown that increasing the selection pressure during phage display selects for VHHs with increased stability. Comparison of the sequences of the VHHs selected under application conditions with a large number of sequences of VHHs obtained after selection for under physiological conditions revealed that an arginine was often observed at position 44 in the more stable VHHs. Point mutations of position 44 confirmed the importance of a basic amino acid at this position.

Materials and Methods

Strains and growth media

The *Escherichia coli* strain used in this study for phage display and plasmid propagation was *E. coli* TG1. This strain was grown in 2TY medium with 100 µg/ml ampicillin and/or 25 µg/ml kanamycin and/or 1% (v/v) glucose when appropriate.

VHHs were produced in *Saccharomyces cerevisiae* VWK18 gal1 (40) as described before (44). In short, individual *S. cerevisiae* colonies were transferred to test tubes containing selective minimal medium (comprising 0.7% yeast nitrogen base, 2% glucose, supplemented with the essential amino acids and bases) and were grown for 24 h at 30°C. Subsequently, the cultures were diluted ten times in YP medium (comprising 1% yeast extract, 2% bacto pepton) and 2% glucose and 2% galactose for induction. After 48 h of induction with galactose, the cells were harvested. Antibody fragments were purified with a ProtA column or with TALON (Clontech) using the HIS-tag.

The *Malassezia furfur* extract was obtained from John Whitley from the Karolinska hospital in Stockholm, Sweden. Preparation of this extract has been described by Zargari *et al*. (48). The recombinant Malf1 (rMalf1) was produced in *Escherichia coli* JM109-DE3 and purified from inclusion bodies using standard protocols (41).

Induction of a humoral immune response in llama.

A llama was immunised with extract of *Malassezia furfur* and recombinant Malf1 in oil emulsion (1:9 V/V, antigen in PBS : Specol) (2) subcutaneously and intramuscularly. Per immunisation round 0.75-1.5 ml water in oil emulsion was injected containing 1 mg *M. furfur* extract or 200 µg recombinant Malf1 protein. The immune response was monitored by titration of serum samples in ELISA with rMalf1 immobilised on Nunc maxisorb plates (coat solution 5 µg/ml rMalf1 diluted in phosphate buffered saline, 100 µl/well). Subsequently, wells were blocked (4 % Marvel in PBS, 400 µl/well) and incubation with serum dilutions in 0.15 % Tween 20/PBS. The bound llama antibodies were detected with poly-clonal rabbit-anti-llama IgG (R906, ID-DLO) in 0.15 % Tween 20/PBS, 100 µl/well (obtained via immunising rabbits with llama immunoglobulins purified via ProtA and ProtG columns) and swine-anti-rabbit immunoglobulins (Dako) conjugated to horse radish peroxidase (in 0.15 % Tween 20/PBS, 100 µl/well). Finally, the peroxidase enzyme-activity was determined with tetramethylbenzidine (TMB) and ureaperoxide (UP) as substrate and, after termination of the reaction by adding H_2SO_4, the optical density was measured at 450nm .

Isolation of VHH fragments

A blood sample of about 200ml was taken from an immunised llama. An enriched lymphocyte population was obtained via centrifugation on a Ficoll (Pharmacia) discontinuous gradient. From these cells, total RNA was isolated by acid guanidium thiocyanate extraction (5). After first strand cDNA synthesis using MMLV-RT (Gibco-BRL) and random oligonucleotide primers (Pharmacia), DNA fragments encoding VHH fragments and part of the long or short hinge region were amplified by PCR using specific primers: V_H - 2B: AGGTSMARCTGCAGSAGTCWGG (S = C and G, M = A and C, R = A and G, W = A and T), Lam-07: AACAGTTAAGCTTCCGCTTGCGGCCGCGGAG CTGGGGTCTTCGCTGTGGTGCG (short hinge), Lam-08: AACAGTTAAGCTTCCGC TTGCGGCCGCTGGTTGTGGTTTTGGTGTCTT GGGTT (long hinge).

The DNA-fragments generated by PCR were digested with *Pst*I (coinciding with codon 4 and 5 of the VHH domain, encoding the amino acids L-Q) and *Bst*EII (naturally occurring in FR4 region of the majority of the VHH-genes and coinciding with the amino acid sequence Q-V-T). The digested PCR-products, with a length between 300 and 400bp (encoding the complete VHH domain, but lacking the first and the last three codons), were separated by electrophoresis on agarose gels and, after purification from the gel-slices with the Qiaex-II extraction kit (Qiagen), cloned (*Pst*I/*Hin*dIII) in the phage display vector pUR4676. This vector was derived from pHEN1 (17).

Selection of VHHs binding Malf1

From the isolated RNA of two different bleeds, which were taken after 3 immunisations with the *Malassezia furfur* extract and after 6 immunisations with the recombinant Malf1 protein phage-display libraries were created. Each library was composed of two sub-libraries, one containing DNA fragments derived from short-hinged antibody fragments and one derived from long-hinged antibody fragments (28). After phage production from each sub-library, phages were isolated and pooled.

A display library with 10^7 clones, of which 75% is estimated to contain a complete VHH encoding insert (Van der Vaart, personal communication), was constructed in phagemid vector pUR4676. Phage particles exposing VHH fragments were prepared by infection of *E. coli* TG1 cells harbouring the phagemid with helper phage VCS-M13

(Pharmacia). Free VHHs were removed by precipitation of phage from the culture supernatant with PEG6000, thereby avoiding a competition for binding to antigen between phage-bound and free VHH domains. The *E. coli* phage was incubated for one hour in a maxisorp tube with immobilised rMalf1. The phage particles complexed to rMalf1 via their exposed VHHs were, after 20 washes with PBS, eluted with a pH-shock (0.1 M triethylamine). This phage population, which is enriched for Malf1 binders, was rescued by infection of *E. coli* host cells (24). Individual *E. coli* clones were grown in wells of microtiter plates, and the production of VHHs was induced by addition of IPTG (0.1 mM). Culture supernatants containing free VHHs were tested in ELISA experiments for binding rMalf1 using the myc-TAG for detection. OD450 signals >0.2 (twice above background) were considered positive for binding to Malf1.

Selection of VHHs binding Malf1 via phage display under application conditions.

The shampoo stability of Malf1 in the ELISA was tested by incubating the plates with various shampoo concentrations. Various concentrations of Andrelon™ (10%-40%) and Organics™ (2%-20%) were used. After washing, all ELISA plates were still immunoreactive.VHH selection under application conditions was performed in 2-40% (v/v) Andrelon™ which will be called shampoo-1 and in 2-20% (v/v) Organics™, which will be called shampoo-2. The chemical composition of these shampoos is available as supplemental material (at http://www.cmbi.kun.nl/articles_ext/). Dilutions of shampoo were made in PBS with 0.1 % (w/v) Marvel and 0.05% (v/v) Tween20. Selections were performed as described above. The binding step in the phage display was 1 hour and at room temperature. Individual *E. coli* clones were grown and the production of VHH was induced by addition of IPTG (0.1 mM). Culture supernatants containing free VHHs were tested in ELISA experiments in 10% shampoo-1, or 2% shampoo-2 for binding rMalf1 using the myc-TAG for detection. OD450 signals >0.2 were considered positive for binding to Malf1.

ELISA

Maxisorp plates were coated overnight with 10 µg rMalf1 in 100µl/well at 4°C. Plates were blocked with 200 µl 4% Marvel in PBS. Equal amounts of VHH (3.3 µM) were incubated for 1 hour at room temperature in 2% Marvel, 0.05% Tween 20 in PBS or in the desired concentration of shampoo-1, shampoo-2, ureum, or guanidine/HCl diluted in PBS, 0.5% Marvel, 0.05% Tween 20 (100µl/well). After washing with PBS/Tween (0.05%), the plates were incubated with poly-clonal rabbit-anti-llama IgG and swine-anti-rabbit immunoglobulins (Dako) conjugated to horse radish peroxidase (in 0.05 % Tween 20/PBS, 100 µl/well). Finally the peroxidase enzyme-activity was determined with tetramethylbenzidine and ureaperoxide as substrate. The optical density was measured at 450nm after termination of the reaction by adding of 50 µl 1M H_2SO_4. All experiments were done at least in duplicate, typical results are shown.

Construction of the mutants

Mutants of Malf A7 and Malf D12 were created with PCR by 'splicing by overlap extension' (16). In short, complementary primers, with nucleotides substituted for generating Arg44, Lys44, or Glu44 respectively, are used in a two step PCR to generate DNA fragments containing the desired mutation. These products were digested with restriction enzymes and cloned into the original vector. The correctness of the mutated genes was confirmed by sequence analysis. All numbering and CDR definitions according to Kabat (19)

Crystallisation conditions

Crystals were obtained in 10% PEG 6000, 100 mM Hepes (pH 7.3) and 5% MPD using the vapor diffusion method. The drop was constituted from 1 µl of protein at 11-15 mg/ml and 1 ml of reservoir solution. The OE7 crystals pertain to the trigonal space group $P3_221$ with unit cell dimensions a=b=71.40 Å, and c=74.84 Å. They contain a molecule per asymmetric unit giving (assuming a molecular mass of 12500 Da) a Vm= 4.4 $Å^3$/Da which correspond to a solvent content of 72 % (25). Crystals were cryoprotected with 7% MPD and flash frozen to 100 K.

Data collection and processing

The 2.2 Å resolution data were collected on the beamline ID14-EH2 (ESRF, Grenoble) using a CCD detector (λ=0.9326 Å). The data were indexed and integrated using Denzo (31), scaled using SCALA and structure factors amplitudes were calculated using TRUNCATE (4). The data processing statistics to 2.2 Å resolution are given as supplemental material in table 1.

Phasing, model building and refinement

The crystal structure of the lama OE7 fragment was solve with the molecular replacement method with the AmoRe program (30) using the lama HC-V fragment (PDB code: 1HCV) as the search model. A solution was obtained giving a correlation coefficient of 0.45 and R-factors 40% and the electron density map obtained was readily interpretable.

Preliminary refinement was performed with CNS (3) against the high (2.2 Å) resolution data set using bulk solvent correction and standard CNS protocols. Cycles of unrestrained, maximum likelihood refinement, and TLS refinement using REFMAC (4) were alternated with manual inspection of the model using the program Turbo-Frodo (39). Final refinement data are summarized in Table 1 at http://www.cmbi.kun.nl/articles_ext/.

The complete model has a R_{work} of 19% and an R_{free} of 20.6% (Table 1). The stereochemistry was analyzed with Procheck (21) which indicates that 95% residues are in the most favorable region and 5% in the additionally allowed region.

Results

Initial selection of VHHs that can bind Malf1

The immune response of an immunised llama was followed by titration of serum samples in ELISA. The results show an enrichment in antibodies against Malf1 after three immunizations. A VHH phage display library with 10^7 clones was created. After production in *E. coli*, the phages were isolated from the medium. The results of two rounds of phage display with selection for Malf1 are shown in table 1. In this stage PBS was used during the binding step, and not yet shampoo. The three parallel experiments of the second selection step were all done with the output of experiment C of the first step.

	Coating	*Phage titer*	*Enrichment*	*Positives*	*Perc. positives*
First	A: No coating	3.0×10^3	N.A.	N.D.	N.D.
round	B: 5 µg Malf1	9.3×10^5	310 fold	N.D.	N.D.
	C: 50 µg Malf1	6.6×10^6	2200 fold	11/64	17%
Second	C1: No coating	3.0×10^3	N.A.	N.D.	N.D.
round	C2: 0.5 µg Malf1	1.8×10^8	60.000 fold	4/64	6%
	C3: 5 µg Malf1	1.5×10^9	500.000 fold	21/64	33%

Table 1. Phage titers, enrichments, and percentage of binders of the two rounds of selection for Malf1 binding.

Nearly two hundred monoclonal VHHs were expressed and the Malf1 binding of these fragments was tested in ELISA experiments. The percentage of VHHs capable of binding Malf1 was determined (see table 1). Ten of these were selected and tested for Malf1 binding under application conditions. Figure 1A shows that they all can bind Malf1 in PBS. Figures 1B and 1C show that only one VHH can bind Malf1 in shampoo.

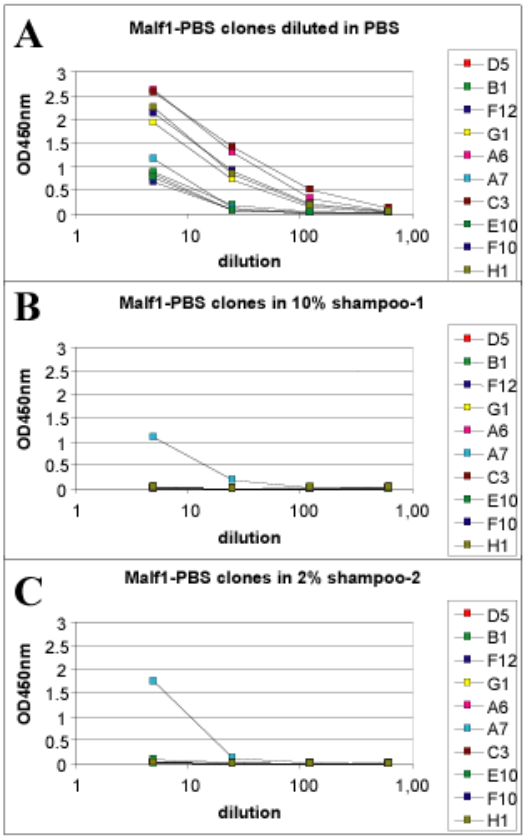


Figure 1 Binding of VHH to Malf1 after 2 selection rounds. A) binding in PBS, B) binding in 10% shampoo-1 C) binding in 2% shampoo-2. Binding was measured for four different dilutions of ten VHHs each.

Phage display in shampoo

Table 2 shows the results of two rounds of phage display with selection for Malf1 in the presence of shampoo. The first round of selection in shampoo was started with the output of experiment C (see table 1). Monoclonal VHHs were expressed and the Malf1 binding of these VHHs was tested in their respective shampoo (see table 2). Ten positive VHHs were picked from F2 and ten from G3.

	Coating	*Incubation*	*Phage titer*	*Enrichment*	*Perc. positives*
First round shampoo	D1: No coating	2% Shampoo-2	3.0×10^4	N.A.	
	D2: 5 µg Malf1	2% Shampoo-2	1.0×10^6	30 fold	55%
	D3: 5 µg Malf1	5% Shampoo-2	6.0×10^4	2 fold	
	D4: 5 µg Malf1	20% Shampoo-2	-	None	
	E1: No coating	5% Shampoo-1	7.5×10^4	N.A.	
	E2: 5 µg Malf1	5% Shampoo-1	1.0×10^6	13 fold	
	E3: 5 µg Malf1	10% Shampoo-1	6.7×10^5	10 fold	10%
	E4: 5 µg Malf1	40% Shampoo-1	7.5×10^4	None	
Second round shampoo	F1: No coating	2% Shampoo-2	3.0×10^3	N.A.	
	F2: 5 µg Malf1	2% Shampoo-2	2.6×10^7	8600 fold	82%
	F3: 5 µg Malf1	5% Shampoo-2	5.1×10^4	17 fold	
	F4: 5 µg Malf1	20% Shampoo-2	-	None	
	G1: No coating	5% Shampoo-1	3.0×10^3	N.A.	
	G2: 5 µg Malf1	5% Shampoo-1	1.2×10^6	400 fold	
	G3: 5 µg Malf1	10% Shampoo-1	1.2×10^6	400 fold	60%
	G4: 5 µg Malf1	40% Shampoo-1	9.0×10^4	30 fold	

Table 2. Phage titers, enrichments, and percentage of binders of the two rounds of selection for Malf1 binding in shampoo.

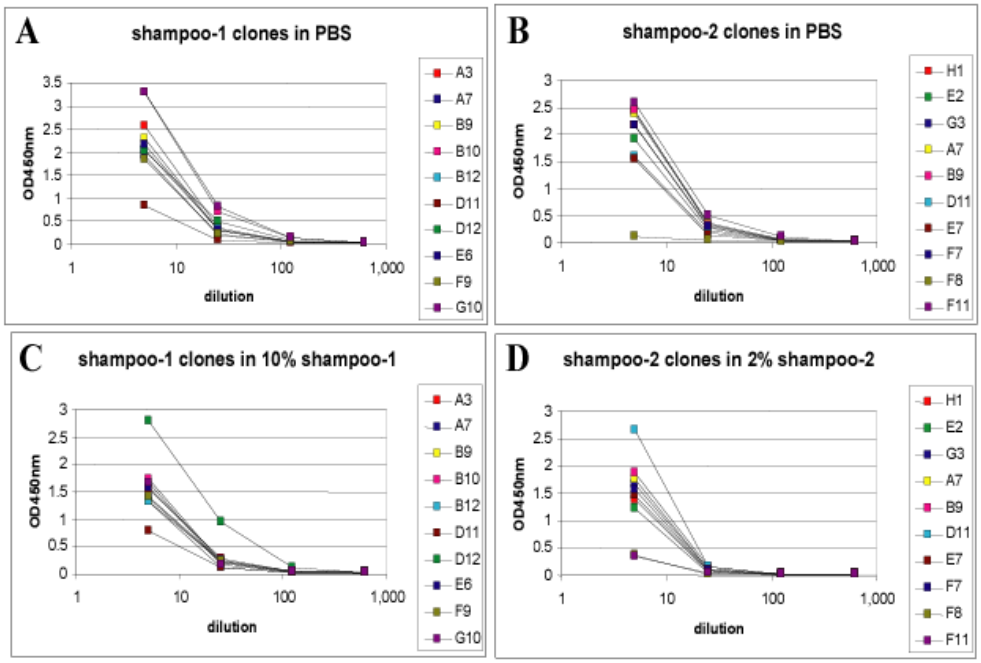


Figure 2. Binding of VHH to Malf1 after 2 selection rounds in shampoo. A) binding of shampoo-2 selected VHHs in PBS B) binding of shampoo-1 selected VHHs in PBS C) binding of shampoo-2 selected VHHs in shampoo-2 D) binding of shampoo-1 selected VHHs in PBS binding in 10% shampoo-2. Binding was measured for four different dilutions of ten VHHs each.

These 20 VHHs were tested for binding to Malf1 in PBS, and in the respective shampoo used for their selection (figure 2). Eight of the ten shampoo-1 selected VHHs and eight of the ten shampoo-2 selected VHHs all bind Malf1 roughly equally well in their respective selection shampoo. In both cases one VHH binds significantly worse, and one VHH binds significantly better. All but one shampoo-selected VHHs can bind Malf1 in PBS, indicating that selection in shampoo normally does not lead to VHHs that require shampoo for binding.

Figure 1 showed that only one of the PBS-selected VHHs could bind Malf1 in shampoo. Figure 2 showed that all shampoo-selected VHHs could bind Malf1 in shampoo, and that all but one of the shampoo-selected VHHs could bind Malf1 in PBS. Figure 3 shows the Malf1 binding of the 20 shampoo-selected clones in different concentrations of their respective shampoos, and the Malf1 binding of the ten PBS-selected clones in different concentrations of the two shampoos. It can be seen that all shampoo-selected VHHs bind Malf1 in their respective shampoos and that the one PBS-selected VHH that

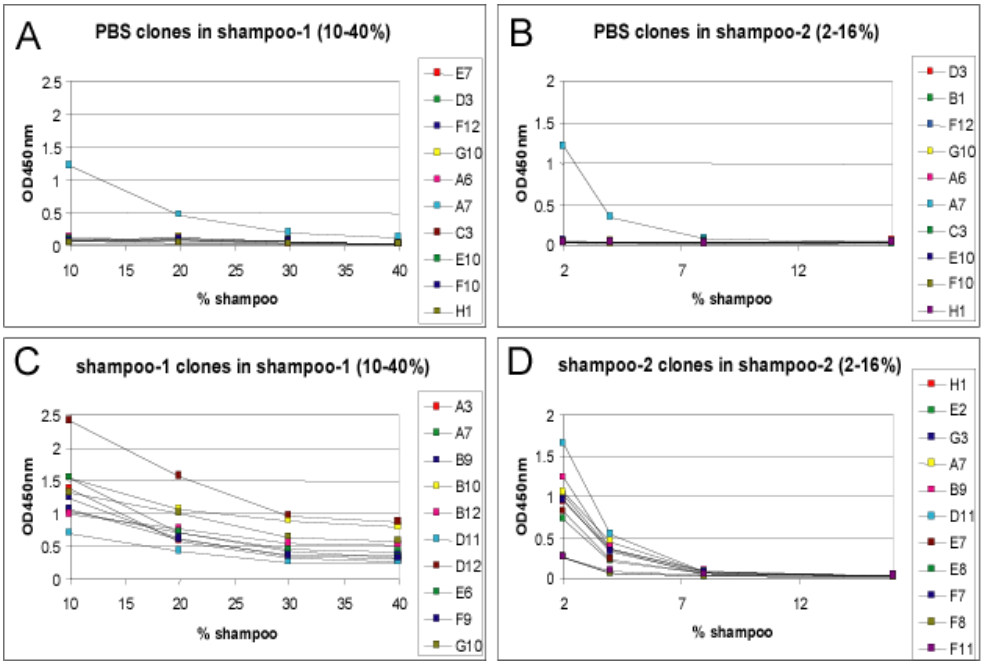


Figure 3. Malf1 binding by VHHs in the presence of shampoo. A) PBS-selected VHHs in shampoo-1 B) PBS-selected VHHs in shampoo-2 C) shampoo-1 selected VHHs in shampoo-1 D) shampoo-2-selected VHHs in shampoo-2.

can bind Malf1 in shampoo does so equally well as the shampoo-selected VHHs except at higher concentrations of shampoo-1.For unknown reasons, it is easier to select for VHHs that can bind Malf1 in high concentrations shampoo-1 than in high concentrations shampoo-2. We therefore concentrated our attention more towards shampoo-1 than towards shampoo-2 during the remainder of our research. This choice was mainly based on the fact that high concentrations (i.e. 10-25%) of shampoo are applied upon washing ones hair and the skin of the head.

Crystal structure

The crystal structure of a shampoo-selected VHH (OE7) was determined in order to investigate whether the structure of the shampoo-selected VHHs was different from VHHs selected under physiological conditions.The Malf OE7 VHH structure is close to that

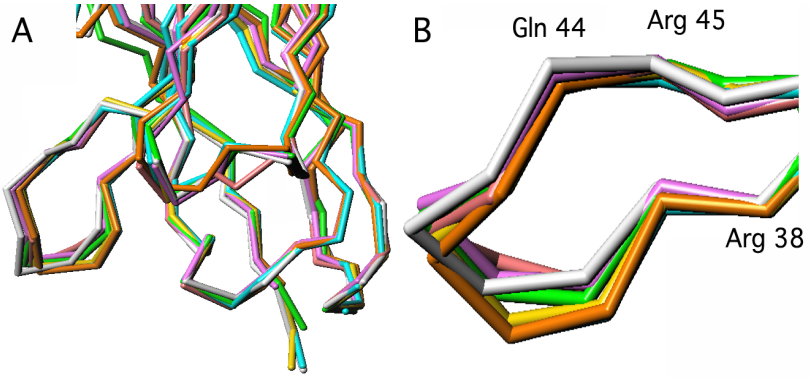
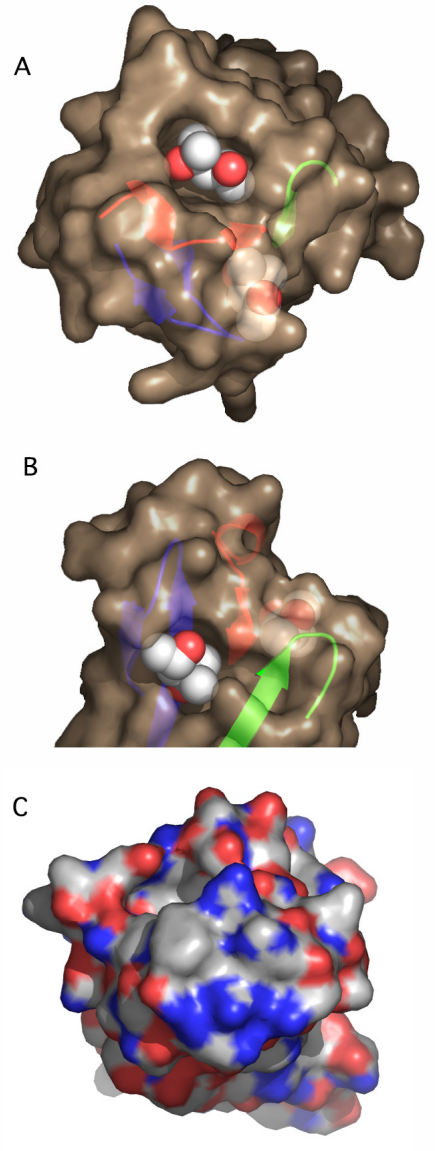


Figure 3. Conformation of the Malf OE7 VHH compared to other VHHs. A) The overall fold of the framework. The CDRs area has been removed for clarity. B) The compared conformation of the loop 38-46.



of other VHHs, besides for the three CDRs (Figure 4a). In particular, the loop 39-45 has a very well conserved conformation

(Figure 4b). Indeed, the precise epitope of Malf OE7 is unknown. In the crystal structure, however, two crevices are well identified close to the CDRs. The first one is located on top of the VHH (Figure 5a), between CDR1 and CDR2, and contains a serendipitous MPD molecule from the crystallization liquor. The second one, which is located on the VHH side, between CDRs 1 and 3, and close to the beginning of CDR2 (Figure 5b), also contains a MPD molecule. Above these two crevices, two stretches of residues formed by CDRs 1 and 3 protrude from the VHH body. These

Figure 4. The molecular surface of Malf OE7, with the 3 CDRs displayed in red, green and blue, respectively. A) Top view of the combining area with a MPD molecule bound. B) Side view of the VHH, with the second MPD molecule bound. C) Top view of the VHH, with the positively charged patch (blue) formed of CDR1 and CDR3.

stretches are particularly charged positively (Figure 5c), since they bear Arg 30, 97 and 99, as well as His 98 (Figure 5c).

Conformation of the loop 38-45 is very well conserved, whatever residues in position 44 are. Gln 44 points towards the solvent in Malf OE7, as well as all the side chains in the other VHHs. VHH-R9 bears a Gln residue, as Malf OE7, but VHH-R2, Car24, amy-B07 and amy-D08 position 44 bears a Glu residue, while Amy-D10 bear a Gly. Position 45 always contains an Arg residue, except for amy-B07 which bears a Cys residue, forming a disulfide bridge with its CDR3 (8). None of these substitutions, however, influence the loop nor the framework conformations.

Sequence analysis

The shampoo-1-selected VHH-D12 binds Malf1 significantly better at high shampoo concentrations than the 29 other VHHs that were tested. However, the Malf1 binding of VHH-D12 in PBS is normal compared to all other VHHs. This suggests that VHH-D12 has one or more mutations beneficial for Malf1 binding in shampoo. The sequences D12, A7, and 6 PBS-selected and 11 shampoo-selected good Malf1 binding VHHs were determined (see figure 6).

Not surprisingly, the variability among the PBS-selected sequences is considerably higher than the variability among the shampoo-selected ones, especially in the CDRs (see figure 6). The CDRs of the shampoo-selected VHHs are on average more hydrophilic than the CDRs of the PBS-selected ones. This might suggest that the hydrophobic tails of some of the soap-like shampoo components shield interactions of antibodies that recognize more hydrophobic epitopes.

A sequence comparison reveals five positions where D12 differs from a significant number of the other shampoo-selected VHHs (see figure 6). Three of these positions are located in CDRs, which make them bad candidates for further analysis in mutation studies. Two of these positions, R44 and K75, are located in the framework. In 684 VHH sequences, determined in our other, unrelated experiments on llama antibody fragments, we found only seven times an arginine at postion 44, and 528 times a lysine at position 75. This suggests that R44 might be (partially) responsible for the good Malf1 binding in shampoo. The PBS-selected VHH-A7 binds Malf1 reasonably well in shampoo. A7 differs at only three sequence positions from D12 (see figure 6). Two of these positions are

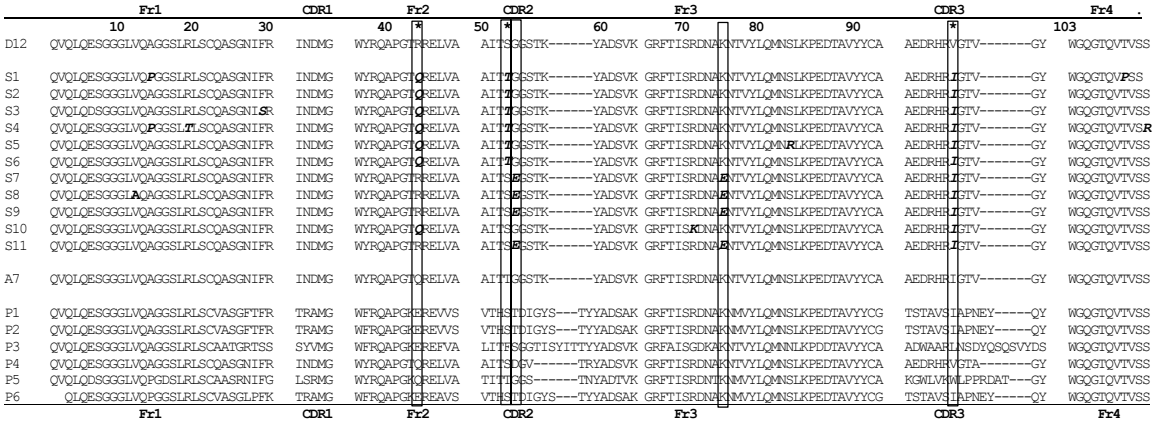


Figure 6. A) Sequences of VHHs. S1-9 are VHHs that bind Malf1 well in shampoo-1 and S10-11 bind well in shampoo-2. P1-6 bind Malf1 well in PBS. D12 is the best shampoo-selected Malf1 binder in shampoo, and A7 is the best PBS-selected Malf1 binder in shampoo. CDRs and framework regions are indicated. The five positions where D12 differs from many shampoo-selected VHHs are boxed. The three positions where A7 differs from D12 are indicated with an asterix.

located in CDRs, and the third is position 44, which is a Gln in A7. These sequence analyses point at an important role for residues at position 44 in the Malf1 binding of VHHs in shampoo. To study this effect further, the residues at position 44 in D12 and A7 were exchanged. Figure 7 shows the Malf1 binding results of the A7 and D12 wild-type VHHs and the A7-Q44R and D12-R44Q mutants. A clear correlation is seen between the presence of arginine at position 44 and Malf1 binding. However, in shampoo this effect is much stronger than in PBS.

To investigate whether the stability caused by R44 was shampoo-specific or more general, Malf1 binding of the four VHHs shown if figure 7 was tested in the presence of guanidine HCl and urea. Figure 8 shows the result of guanidine HCl and urea titrations of VHH-Malf1 binding. In both denaturants the Malf1 binding is better for the R44 variants than for the Q44 variants. No binding is observed for any of the four variants above 2 M guanidine HCl, or 2.5 M urea, except for A7-Q44R that is still binding Malf1 at 3.5 M urea.

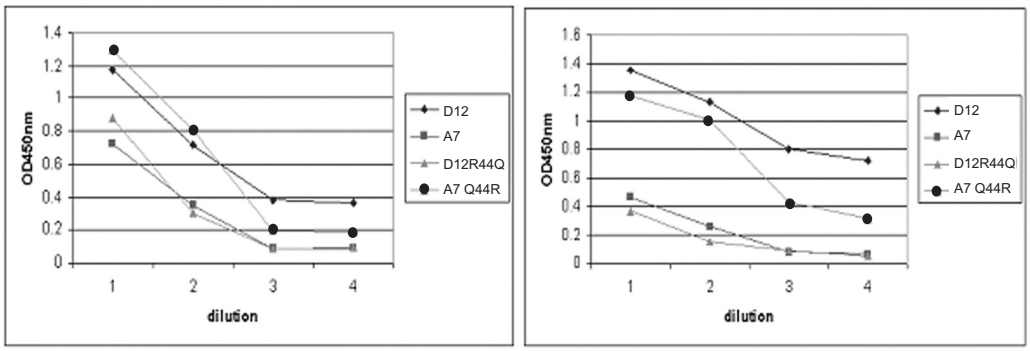


Figure 7. Malf1 binding of wild-type and mutant A7 and D12. A) in PBS. B) in shampoo.

In order to shed light on the enhanced Malf1 binding of VHH variants with an arginine at position 44, we also produced the variants A7-Q44K and D12-R44K. The rationale behind this experiment is that arginine and lysine both are positively charged residues so that these variants potentially could answer the question whether the enhanced Malf1 binding in shampoo is caused by the introduction of a positive charge, or by specific characteristics of arginine. A lysine and an arginine are structurally comparable, however an arginine has a relatively rigid side chain, while lysine has a relatively flexible side chain. At pH 11, arginine is expected to be positively charged, whereas lysine is expected to be only partially charged at this pH.

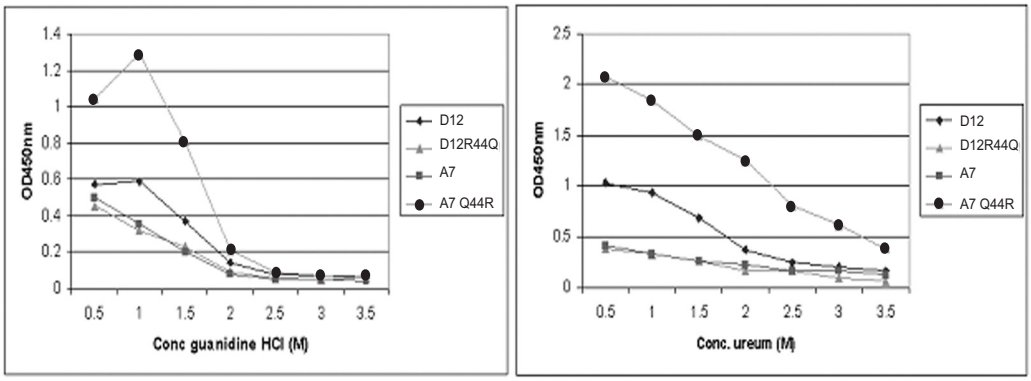


Figure 8. Malf1 binding of wild-type and mutant A7 and D12 A) in guanidine HCl B) in urea

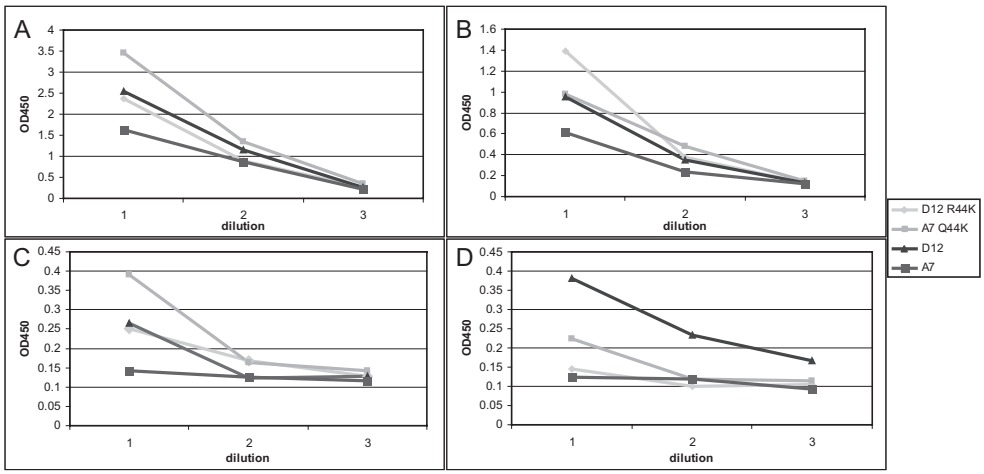


Figure 9. Malf1 binding of wild-type A7, D12, mutant A7 Q44K and mutant D12 R44K. A) in PBS at pH 7 B) in shampoo at pH 7C) in PBS at pH 11 D) in shampoo at pH 11.

Figure 9 shows that Malf1 binding of the Q44, K44 and R44 VHH variants is significantly reduced at pH 11 in both PBS and shampoo-1. This is not a surprise, as proteins tend to be less stable at this pH. At pH 11 the R44 variants still bind Malf1 very well in 10% shampoo-1, while the Malf1 binding of the K44 variants is reduced by about a factor of two. In PBS, on the other hand, the reduction in the binding of Malf1 is about the same for all four variants. This experiment strongly suggests that the enhanced Malf1 binding in shampoo is caused by the introduction of a positive charge at position 44.

Discussion

Phage display was successfully performed for the isolation of Malf1 specific VHH antibody fragments. Although a lot of VHHs show binding to Malf1 under physiological conditions, most of them were shown not to bind Malf1 in the presence of shampoo. As Malf1 binding is also strongly reduced in the presence of denaturants like urea or guanidine HCl, it seems likely that stability of the VHH is the main determinant for Malf1 binding.

We used phage display in shampoo to select a series of VHHs that can work well in shampoo. It has been observed many times that mutations that protect a protein against one type of external stress will also protect the protein against other forms of external stress

(11, 32). Indeed, the VHH variants that were selected to work well in shampoo also showed enhanced resilience against urea and guanidine HCl.

It was possible to perform the phage display in the presence of high concentrations of shampoo to select VHHs that can function well in shampoo. It was surprising that this whole procedure worked under such harsh conditions without any major modifications to the standard phage display protocols. The growing interest in industry for antibody based applications generates a demand for rapid selection and isolation of antibody fragments which have high affinity and are stable in the environment in which they should perform their function. This paper shows that selection pressure can improve VHH performance in a predictable direction. This will eradicate time-consuming improvement of the antibody fragments by mutagenesis.

The phage display helped us detect a series of VHHs with enhanced binding properties. The variability within these sequence families gives a good impression of the possible motifs involved in protein stability and binding. Using multiple sequence alignment analyses of VHH families one quickly gets good suggestions for site directed mutagenesis experiments that can either explain the observations or further improve the obtained VHH variants.

From the analysis of the sequences of 12 shampoo-selected and 7 PBS-selected VHHs, we concluded that an arginine at position 44 is important for proper VHH functioning in shampoo. This was confirmed by mutagenesis studies. Introduction of a lysine at position 44 had equally beneficial effects, indicating that the positive charge is more important than arginine specific characteristics. This is somewhat surprising in light of the observation that lysine was never observed at position 44 in the VHH variants analysed in this study, and only 4 times in 684 VHH variants that we sequenced in previous selection studies that had no relation to shampoo. So, even though selection by phage display can quickly produce good VHH variants, classical mutagenesis work can still add more information.

The crystal structure of a shampoo-selected VHH was determined. The influence of the Gln->Arg mutation is most probably an electrostatic effect. This is supported by the fact that a Lys residue induces the same stabilization effect. Indeed, one should keep in mind that the harsh medium in which the llama antibodies are raised contains a high concentration of negatively charged molecules. This fact might explain the charged apex

CDR1 and 3 of the Malf OE7 VHH, as well as the positive effect of the framework mutation. The fact that this residue is barely observed as naturally occurring, indicates selection of a rare subpopulation of VHHs, induced by the harsh medium conditions.

The proof of the pudding is in the eating. Obviously, the final goal of this project is the production of VHHs that can be added to shampoo to reduce dandruff. We have produced VHHs that can bind Malf1 under realistic shampoo conditions. It is still to be proven that this binding will also lead to neutralization of *Malassezia furfur* on the scalp. However, these VHHs do bind Malf1. So, even if they do not neutralize *Malassezia furfur* they can still be used to deliver anti dandruff agents to the surface of *Malassezia furfur*.

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Chapter 4

Decreasing trypsin susceptibility of single domain antibodies: implications for structural stability

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Submitted to Protein Engineering, Design and Selection

Abstract

The trypsin susceptibility was reduced of a llama single domain antibody fragment (VHH) that can neutralize rotavirus. The antibody sequence revealed eleven potential trypsin cleavage sites. Analysis of a homology model and a multiple sequence alignment identified which of these potential sites were good candidates for mutation studies. Mutants were analyzed on trypsin susceptibility, rotavirus binding affinity, production levels, and thermostability. Mutation of position 27 decreased trypsin susceptibility and increased thermostability. Mutant R27A also showed increased production levels and equal binding affinity compared to the wildtype. These results show that *in silico* stabilization can contribute to functional improvement of a VHH, which in this case might lead to an economically feasible product for neutralization of rotavirus for people in developing countries.

Introduction

Rotavirus is the most common cause of gastroenteritis in young children. It causes high mortality rates in developing countries and is a large economic burden in developed countries (Cook *et al.*, 1990 ; Parashar *et al.*, 2003). The importance of rotavirus has stimulated much research into vaccine development (Cunliffe *et al.*, 2002). Presently, the best vaccine that is available (Joensuu *et al.*, 1997), RotaShield, is associated with intussusception (MMWR, 1999). Therefore this vaccine has been withdrawn from the market (MMWR*, 1999; Miller, 1999).

Several studies have illustrated the feasibility of the use of orally administered antibodies for the inhibition of viral infection (Dagan and Eren, 2003; Burrer *et al.*, 2003). Several groups have reported the inhibition of rotavirus with antibodies (Sarker *et al.*, 2001; Ludert *et al.*, 2002). A large disadvantage of the use of antibodies is that they have to be administered orally. This implies that the antibodies have to pass the gastrointestinal tract where they are exposed to gastric acid, high concentrations of bile salts and other small molecules, and digestive enzymes. Petschow showed that the activity of antibodies in the gastrointestinal is mainly decreased by low pH and trypsin (Petschow and Talbott, 1994).

Rotavirus infections seem independent of the hygienic state and are so widespread that nearly the entire world population will have been infected before the age of five. Almost all of the 440.000 deadly rotavirus infections involve children in developing countries (Parashar *et al.*, 2003), so that a method to prevent infection necessarily must be cheap. This cost argument, at first, seems to eliminate the use of antibodies as a viable approach, particularly in developing countries.

Members of the family of the *Camelidiae*, like camels, dromedaries and llamas have been shown to possess a unique subclass of antibodies lacking light chains (Hamers-Casterman *et al.*, 1993), and consequently have only a single binding domain. This variable domain of a heavy chain antibody is called a VHH. VHHs have several advantages over conventional antibodies, that make them promising tools for a wide range of applications (Van der Linden *et al.*, 1999; Ledeboer *et al.*, 2002; Dumoulin *et al.*, 2003; Verheesen *et al.*, 2003). VHHs are very cheap to produce, because of high production levels in yeast (Frenken, 2000 *et al.*; Thomassen *et al.*, 2002), they can easily be cloned, and display reversible unfolding (Perez *et al.*, 2001; Dolk, manuscript submitted).

A useful VHH must resist low pH and pepsin in the stomach and trypsin in the intestines. The low activity of trypsin at low pH precludes the simultaneous optimisation against these three hazards with simple phage display selection techniques. As there exists little experience in engineering protein stability against low pH, we have used phage display to select VHHs that can survive stomach conditions and neutralize rotavirus *in vivo* (Van der Vaart, manuscript in preparation). In this study we describe the additional protection of one good candidate VHH, VHH1, against trypsin in the intestines by protein engineering.

For most rotavirus strains trypsin is required for cleavage of viral protein VP4 into VP5 and VP8 in order to infect efficiently (Gilbert and Greenberg, 1998). Even though trypsin is essential for rotaviral infection, trypsin inhibitors cannot be used for prolonged protection because trypsin has too many important functions. Trypsin is one of the most abundant proteases in the intestines, and it has been shown that it can decrease the neutralization effect of antibodies dramatically (Blum *et al.*, 1981; Hilpert *et al.*, 1987; Petschow and Talbott, 1994; Pacyna *et al.*, 2001). If prolonged protection against rotavirus is necessary, the antibody fragment should not be susceptible to trypsin. Trypsin cleaves Arg-Xxx and Lys-Xxx bonds. The cleavage efficiency depends on the local sequence as

described by Frenken (Frenken *et al.,* 1993) and on the local three-dimensional structure (e.g. Hubbard *et al.,* 1994). The potential trypsin cleavage sites in VHH1 were analyzed, and the most likely tryptic cleavage sites were removed by mutagenesis. The mutated VHH should favourably compare to the wild-type according to the following criteria:

- Less susceptible to trypsin;
- At least equal production levels;
- At least equal binding affinity;

Normally, all mutations that improve the hydrogen-bonding pattern, increase the buried hydrophobic surface area, add a salt bridge, etc., while not simultaneously introducing strain or other bad side effects, will improve the overall protein stability, independent of their location in the protein. However, if the inactivation process is irreversible, it only makes sense to mutate at, or around the location where the first irreversible step takes place. In case of proteolysis, it has been shown that increased stability can only be obtained by mutations that reduce the first cleavage step (Frenken *et al.*, 1993; Van den Burg *et al.*, 1998).

We determined that the arginine at position 27 (R27) (numbering according to Kabat (Kabat *et al.*, 1991)) is the first tryptic cleavage site in VHH1. Mutation of this arginine to alanine (R27A) leads to a dramatically decreased proteolysis. Additionally, R27A fulfills the aforementioned criteria. This mutant seems a good candidate to start inhibition of rotaviruses in the gastrointestinal tract. Its combined properties allow for the economically feasible production required for large-scale application in developing countries, where rotaviral infection is a big problem.

Materials and Methods

Homology modeling

The structure of VHH1 was modelled based on a multiple alignment of sequences from all available PDB structures and VHH1 sequence using ClustalW. The model was build using the WHAT IF software (Vriend, 1990) based on the structure of the VHH directed against RR6, PDB entry 1QD0 (Spinelli *et al.*, 2000), as described previously (Vriend and Eijsink, 1993). Modelling details at http://www.cmbi.kun.nl/articles_ext/.

Mutant design

The accessibility of the arginine and lysine residues in VHH1 was determined using the surface-accessibility module of WHAT IF (Vriend, 1990). The compliance with the serine protease specificity rules of Frenken was determined as described before (Frenken *et al.*, 1993). Multiple alignments were performed with 703 public and proprietary sequences of VHHs as obtained from previous studies on llama antibody fragments. All numbering and CDR definitions are according to Kabat (Kabat *et al.*, 1991).

Construction of the mutants

Mutants of VHH1 were created with PCR by 'splicing by overlap extension' (Ho *et al.*, 1989). In short, complementary primers, with nucleotides substituted for generating the desired mutation are used in a two-step PCR to generate DNA fragments containing the desired mutation. These products were digested with restriction enzymes and cloned into vector pUR 4585 containing a Myc-tag and a His-tag (Van der Linden *et al.*, 2000).

Strains and growth media

The *Escherichia coli* strain used for plasmid (pUR4585) propagation was TG1. This strain was grown in 2TY medium with 100 µg/ml ampicillin and/or 1% (v/v) glucose when appropriate. Transformation to yeast was performed as described before (Gietz and Woods, 2002).

The VHHs were produced in *Saccharomyces cerevisiae* VWK18 gal1. Individual colonies were transferred to test tubes containing selective minimal medium (comprising 0.7% yeast nitrogen base, 2% glucose) and were grown for 18 h at 30°C. Subsequently, the cultures were diluted ten times in YP medium (comprising 1% yeast extract, 2% bacto pepton) and 2% glucose. 2% galactose was added for induction. After 48 h of induction, the cells were harvested. Antibody fragments were purified with TALON (Clontech) according to the manufacturer's protocol.

Trypsin digestion

Eight µg of VHH was incubated with 20 µl immobilized TPCK trypsin (Pierce) in PBS at 37°C. After 0, 15, 45 and 90 minutes of head-over-head incubation, 10 µl samples were taken. Immediately protein sample buffer was added and the samples were boiled for

5 min. Samples were stored at -20°C until all samples were collected, and subsequently separated with SDS PAGE.

Determination of T_M

CD spectra were measured with a Jasco J-810 spectropolarimeter coupled to a Jasco CDF-426S Peltier thermostatted cell holder. Molecular ellipticity at 200 nm was determined from 25°C to 80°C. The spectral bandwidth was automatically kept at 2 nm, the temperature increment was 0.2 C/step and the accumulation time was 2 C/min. 240 µg/ml VHH in H_2O was used in a 0.2 mm quarz cuvette (Hellma). The spectra manager software (Jasco) was used to analyze the spectra. Typically, the spectra of the VHHs were smoothened and the first derivative was determined.

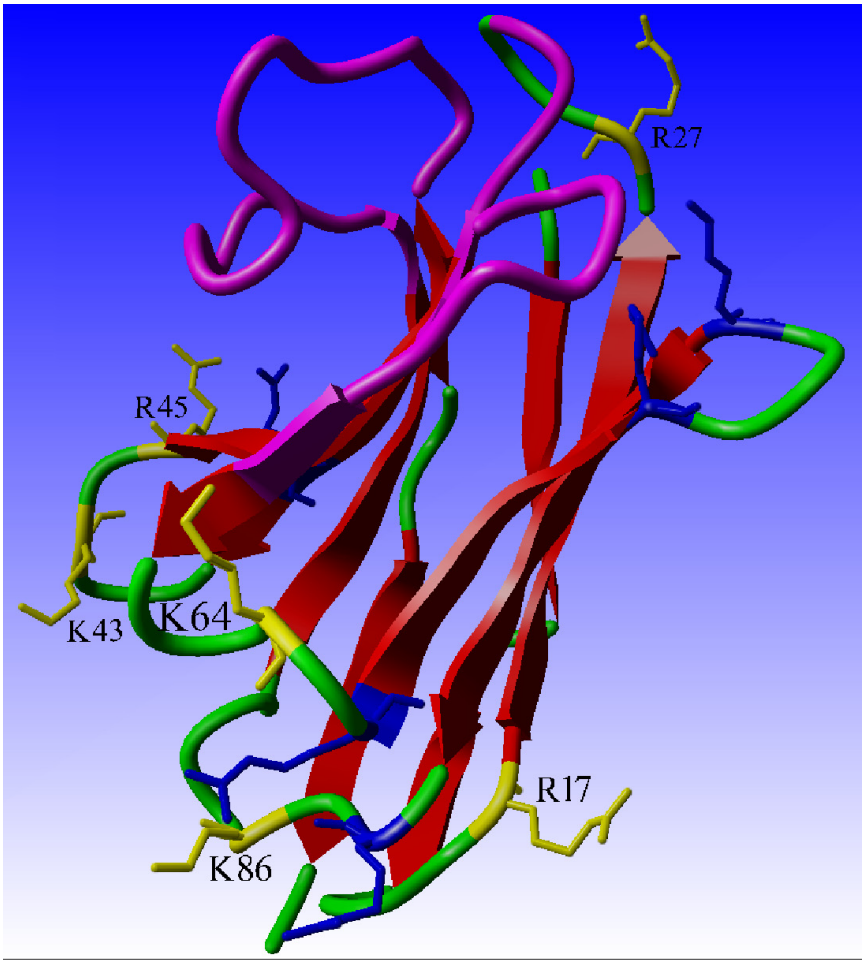
Functional assay

Determination of the binding capacity of the mutants was performed in a competition ELISA based assay. UV-inactivated bovine (Compton) rotavirus strain G3 CK5 was coated onto maxisorp plates. Plates were blocked for 30 minutes with 4 % MPBST (4% Marvel in PBS with 0.05% Tween20). Fifty µl of 250 ng/ml VHH1-biotin in 2% MPBST was added to 50 µl of competition samples. Competition samples contained VHH1 or the mutants. The highest concentration of 50 µg/ml and samples were diluted with 2% MPBST. Subsequently this mixture of biotinylated VHH1 and competitor were incubated for one hour. After washing, streptavidine-HRP (1:1000 in 2% MPBST) was added to all wells and incubated for one hour. Subsequently, the plate was washed and HRP activity was determined with TMB substrate (Biomerieux, Boxtel). The color reaction was stopped with 50 µl H_2SO_4 and measured at 450 nm in a plate reader.

Determination of the binding capacity after trypsin digestion was performed using the same assay. Trypsin digestion was performed with 25 µg VHH1 WT without tags and 25 µg R27A with tags dissolved in 100 µl PBS with 50 µl trypsin beads (Pierce). After 0, 90, 180 minutes and overnight samples of 15 µl were taken, to which 5 µl soy bean trypsin inhibitor (5µg/µl) was added. These VHH samples were diluted to 50 µg/ml in 2% MPBST.

Results and discussion

There are no structures available of anti-rotavirus antibody fragments yet. Therefore the structure of VHH1 was obtained by homology modelling using the VHH



QVQLQDSGGGLVQVGDRLSLSCAASGRTFSSYDMAWFRQAPGKEREFVAAITTSEGTWYG
DAGKGRFTIARVNAKNTVYLHMNRLKPEDTAVYYCAASNQGGSLQISTNYNYWGQGTQV
TVSS

Figure 1. A) Model of VHH1. Strands are represented by red arrows; loop areas by green tubes; the CDRs are purple. The side chains of the eleven arginines and lysines are shown as sticks. The yellow residues were mutated; the blue ones not. B) Sequence of VHH1. The hexapeptides shown in table 1 are underlined. CDRs are in purple. The eleven arginines and lysines are in red, while bold-face indicates the six residues that were mutated.

against hapten RR6 (PDB file 1QD0; Spinelli et al., 2000). One consideration in this template choice was the large number of conserved arginines and lysines between 1QD0 and VHH1. Modelling details such as the list of potential templates, the alignment of template and model sequence, alignment scores, etc., are available at http://www.cmbi.kun.nl/articles_ext/. Figure 1 shows the VHH1 model with the arginines, lysines, and the hyper variable domains (CDRs) indicated. Table 1 lists the sequence patterns around the eleven arginines and lysines. In this table compliance with the serine protease specificity rules of Frenken (Frenken et al., 1993) is indicated.

R17						187.9
V	G	D	**R**	L	S	
+	+	-	+	-	-	
R27						202.3
A	S	G	**R**	T	F	
-	+	+	+	-	+	
R38						50.9
A	W	F	R	Q	A	
-	-	-	+	-	-	
K43						159.3
A	P	G	**K**	E	R	
-	-	+	+	-	+	
R45						177.4
G	K	E	**R**	E	F	
-	+	-	+	-	+	
K64						109.2
D	A	G	**K**	G	R	
-	-	+	+	+	+	
R66						80.1
G	K	G	R	F	T	
-	+	+	+	-	-	
R71						44.6
T	I	A	R	V	N	
-	-	+	+	-	+	
K75						126.9
V	N	A	K	N	T	
-	+	+	+	-	-	
R84						156.6
H	M	N	R	L	K	
+	-	-	+	-	+	
K86						91.2
N	R	L	**K**	P	E	
-	+	-	+	+	+	

Table I:

Compliance with Frenken's sequence rules for serine protease specificity and accessibility. Six residues around each of the eleven trypsin cleavage sites in VHH1 are shown and + and – signs indicate compliance or non-compliance respectively. The right hand column indicates the accessible surface area of the side chains of the arginines and lysines at the P1 position (fourth residue of the six). Residues that were chosen for mutation analysis are boxed.

Frenken's rules for serine protease specificity include four residues before and two residues after the scissile bond. The dominant rule is, of course, that the fourth residue must be an arginine or lysine. Cleavage by trypsin requires local unfolding of the substrate protein to allow proper docking in the active site cleft of trypsin of at least the hexapeptides listed in table 1. Several groups have studied local unfolding processes that preceed irreversible inactivation by proteolytic cleavage. (e.g. Eijsink *et al.,* 1992). Hubbard (Hubbard *et al.*, 1994) used molecular dynamics techniques to study the magnitude of these local unfolding processes, and found that the required unfolding is small in terms of atomic displacement of atoms in the substrate. Vriend and Eijsink (Vriend and Eijsink, 1993), however, found that this small local displacement can only take place under conditions that are close to those required for global unfolding. We reasoned that the degree of local unfolding required for cleavage by trypsin is related to the degree of surface accessibility of the arginine or lysine adjacent to the scissile bond. Table 1 lists the compliance with the Frenken rules and the surface accessibility of the arginines and lysines in VHH1. In figure 2 these results are represented graphically. Since both factors are important for proteolysis, it is to be expected that residues in the upper right corner of the plot are more likely to represent the major cleavage site than residues in the lower left corner.

Based on figure 2 we selected R17, R27, K43, R45, R64 and K86 for mutation. Until now, the relative importance of the Frenken rule compliance on the one hand and structural considerations like the accessible surface areas of the side chains on the other is unknown. The diagonal line drawn in figure 2 is therefore only a qualitative indicator.

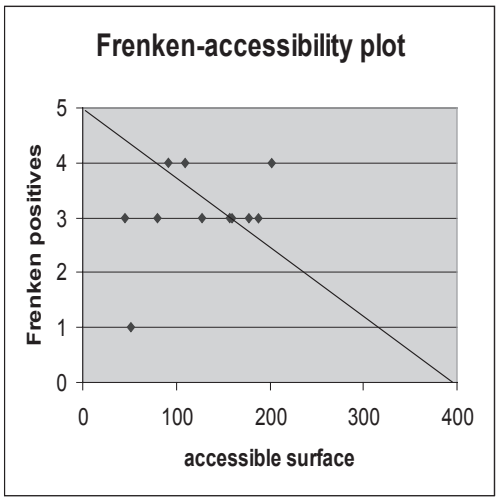


Figure 2. Trypsin cleavage probability of the eleven arginines and lysines in VHH1. The accessible surface area of the side chains is given on the abscise, and the number of residues in the hexa-peptide (see table 1) that comply with the Frenken rules on the ordinate. The diagonal line separates the top right half with likely candidates from the bottom left half with less likely candidates for being the major proteolytic site.

In the absence of other considerations than structural integrity, it is always best to introduce alanines when making mutations, because the introduction of alanines normally does not lead to sterical strain. Except for K43 we mutated all six residues into alanine. A multiple sequence alignment revealed that K43 is highly conserved in VHHs; 87,1% of the 703 sequences have K43, 4.4% have N43, and 2.3% have T43. Other residue types are not observed in a significant number of sequences. T43 was often found in selection experiments designed to find VHHs that are stable in the presence of anionic tensides (Dolk, manuscript in preparation). A combination with the fact that bioinformatic analysis of the VHH1 model showed that asparagines would not fit well at position 43 led to the decision to choose K43T. The alignment also revealed that serine and phenylalanine were observed rather frequently at the positions 17 and 27 respectively. Therefore we decided to also make the mutants R17S and R27F.

One of the criteria was that mutant production-levels in yeast must at least be equally to that of the wild-type. Additionally, secretion to the medium is required to reduce purification costs in a later mass-production stage. The mRNA level of the VHHs was checked on a Northern blot. All mutants show comparable amounts of mRNA (data not shown). Figures 3a and 3b show the production level of the mutant proteins and the wild-type in the intracellular fraction and in the growth media, respectively. These results

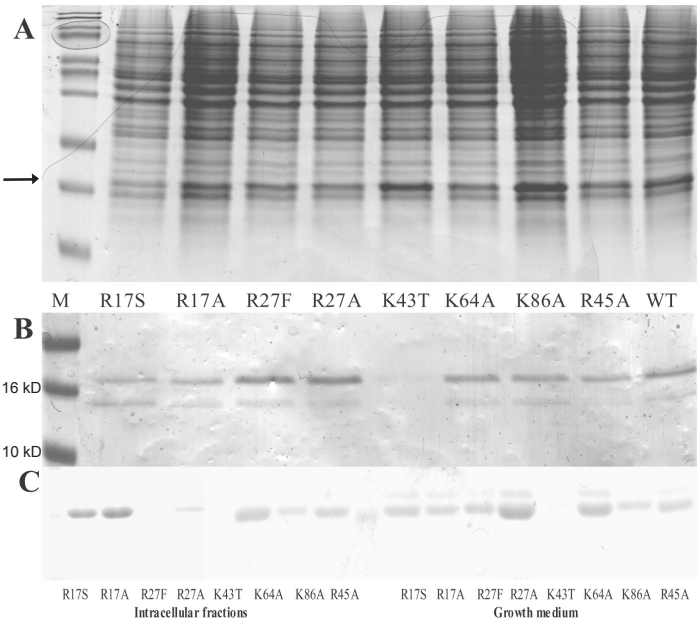


Figure 3: Production and purification of VHH1 mutants. A) intracellular fractions on a Coomassie stained SDS-PAGE gel. B) medium supernatant on a Coomassie stained SDS-PAGE gel. C) purified fractions of the mutants from the intracellular fraction (left) and from the supernatant (right) on a Coomassie stained SDS-PAGE gel.

indicate that mutants R27F and R27A even have higher production levels than the wild-type, and are also better secreted. Mutant K43T is hardly secreted, while R17A and R17S show reduced secretion. It has been shown before that (single) amino acid substitutions can influence the secretion of heterologous proteins by *S. cerevisiae* significantly (Sagt *et al.*, 2000). Figure 3c shows the VHHs that were purified from the intracellular fraction and growth media, respectively. Mutants R17S and R17A are mainly obtained from the intracellular fraction and mutants R27F and R27A mainly from the growth medium. K43T could neither be purified from the intracellular fractions, nor from the growth media and will therefore not be analysed further.

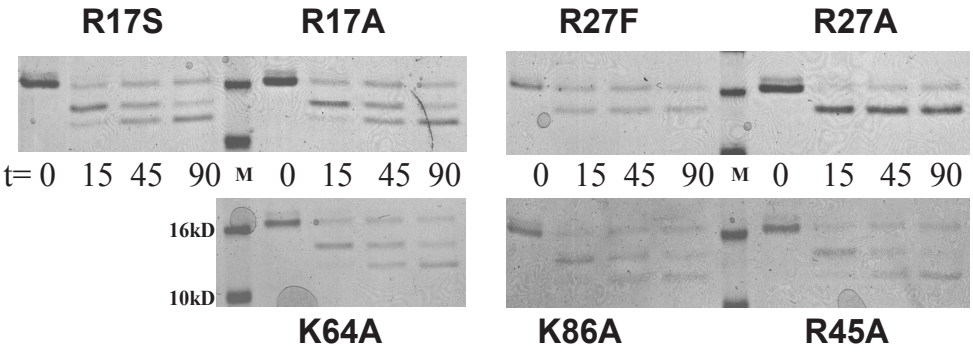


Figure 4 Digests of the mutants before, and 15, 45, and 90 minutes after addition of trypsin on a Coomassie stained SDS-PAGE gel. Protein ladder is indicated with M

The remaining seven mutant proteins were subjected to tryptic digestion. As the second amino acid of the Myc-tag is a lysine, and the tags are likely to be very flexible and thus susceptible to tryptic cleavage.Figure 4 shows that most mutants show three bands on a coomassie stained gel. The upper band is the intact VHH with the Myc-His tag, the middle band is the intact VHH without the tags. This was confirmed by Western blot with anti-his and anti-VHH antibodies (data not shown). The third band is the VHH1 degradation product. The mutants R27F and R27A do not show this third band, indicating that these mutants are not cleaved. The size of the degradation product is about 12 kD, which corresponds well with VHH1 without the first 27 amino acids. Position 27 of VHH1 is thus the first tryptic cleavage site.

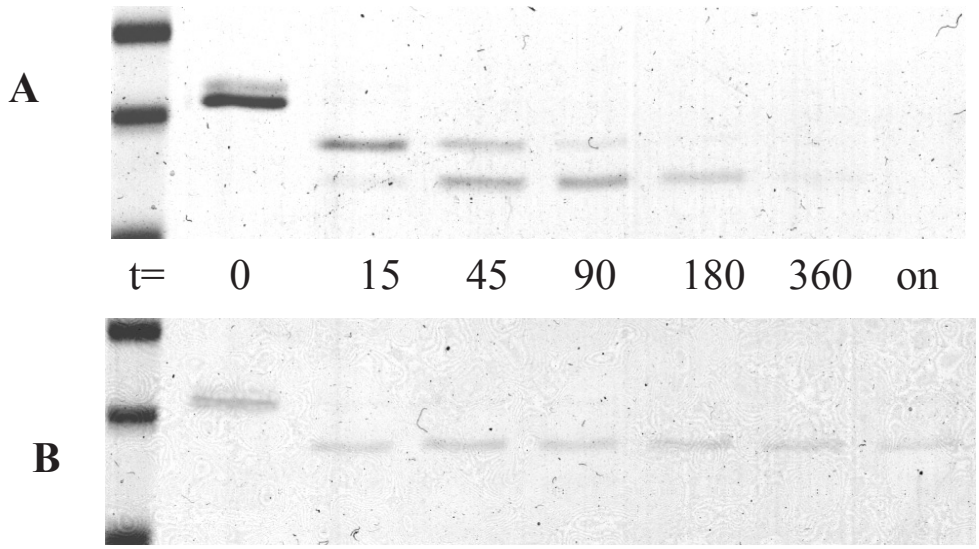


Figure 5 Digestion of wildtype and R27A by trypsin on a Coomassie stained SDS-PAGE gel. A) Tryptic digest of wild-type VHH1 before, and 15, 45, 90, 180, 360 minutes and overnight digestion of trypsin. B) As A, but for R27A.

Figure 5 shows the results of prolonged tryptic digestion of R27A and the VHH1 wild-type. After 45 minutes, 50% of the wild-type is degraded, and after 180 minutes only very little VHH1 is left. Mutant R27A shows complete removal of the tags after 15 minutes. No significant further degradation of R27A is seen up to 24 hours. This assay was only performed on R27A and not on R27F for reasons described below.

A competition ELISA assay was performed to check that the mutants were still able to bind rotavirus, and to estimate their binding affinities. Figure 6 shows the results of competition experiments comparing biotinylated VHH1 and varying concentrations of the non-biotinylated mutants or wild-type. All variants show binding affinities comparable to wild-type, except R27F, which showed a significant decrease in affinity.

It can be seen in the sequence of VHH1 (figure 1B) that cleavage at position 27, as it is just in front of CDR1, does not necessarily influence the integrity of the CDRs under these experimental conditions. Figures 4 and 5 indicate that the degradation product after cleavage at position 27 is present. The question arises whether this first cleavage renders the VHH1 already inactive, or that the inactivation occurs after subsequent cleavages that

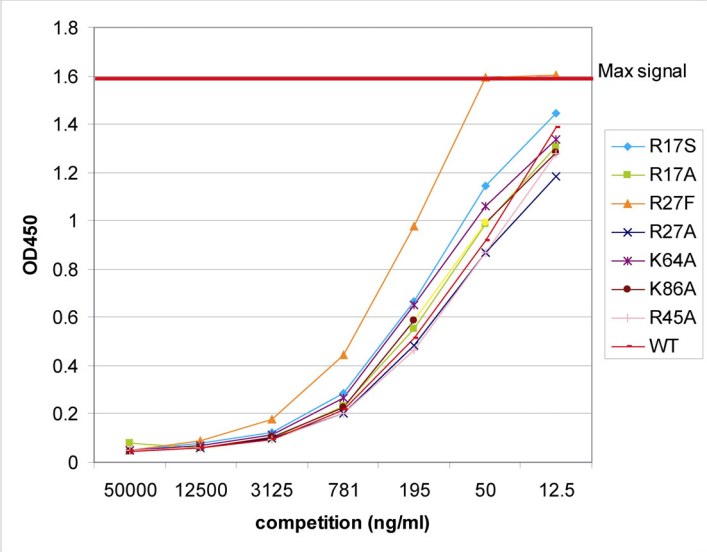


Figure 6: Binding affinity competition experiment. 250 ng/ml biotinylated VHH1 and varying concentrations (as indicated on the horizontal axis) of non-biotinylated variants compete for rotavirus binding. OD450 is related to binding of biotinylated VHH1. The horizontal red line indicates the maximal signal, obtained from biotinylated VHH1 without competition.

are made possible by the initial cleavage at position 27. Figures 5A very clearly shows the degradation of VHH1 as a function of time. Surprisingly, figure 7A and 7C show that rotavirus binding does not decrease appreciably after limited digestion of the first 27 amino acids.

After prolonged digestion, when second site cleavage has occurred (see figure 5) the rotavirus-binding capacity of the wild-type decreases. R27A neither gets cleaved, nor looses its rotavirus-binding capacity under these conditions. So, the partially cleaved VHH1 is still capable of binding rotavirus, and it can thus be concluded that the R27A mutation is

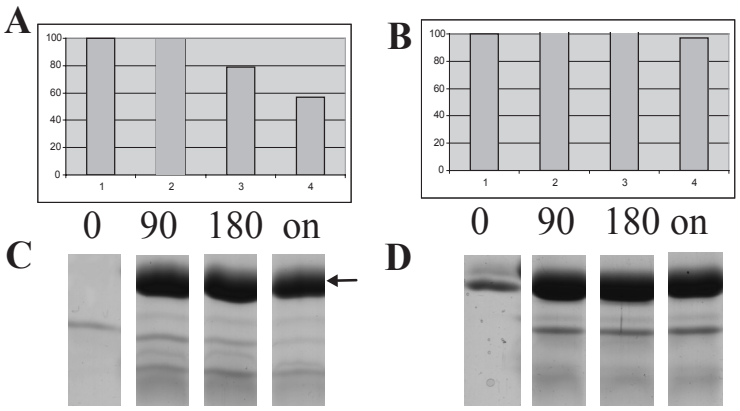
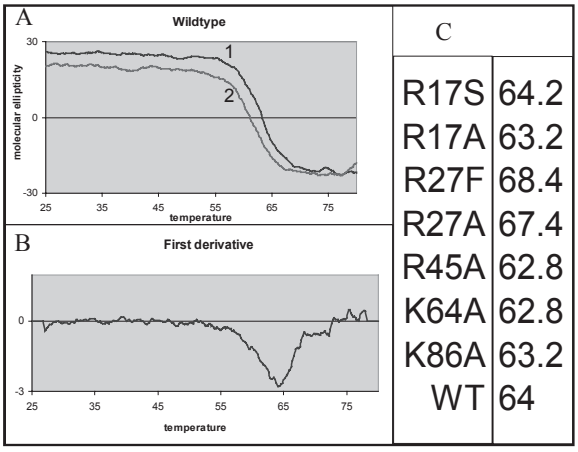


Fig 7. Competition capacity of A) wildtype and B) R27A without trypsin digestion, and after 90 minutes, 180 minutes and overnight digestion with trypsin. Competition capacity before trypsin digestion (0) was set at 100 %. The Coomassie stained gels of samples used for A and B are shown in C and D respectively.The arrow indicates SBTI.

important because it prevents subsequent cleavage at other sites. The binding capacities of the samples used for figure 7 were tested similarly as described for figure 6, but in this case the competition was measured only against one concentration on non-biotinylated material. Trypsin inhibitor did not have any effect on the binding efficiency of biotinylated VHH1 (data not shown). Note that tags are efficiently cleaved from R27A after 90 minutes and this cleavage does not affect binding (figure 7D).

Several groups have indicated that thermal stability of proteins often is also a good indicator for stability against a series of irreversibly inactivating conditions (Parsell and Sauer, 1989; Eijsink *et al.*, 1992). The corollary does not necessarily need to be true. The mutants were designed with avoidance of proteolysis as the major constraint while stability considerations were minor. Figure 8 shows the results of thermal unfolding studies of the wild-type and the seven mutants. The wild-type and the seven mutants all refolded upon cooling. This important feature of VHHs has been observed before (Perez *et al.*, 2001).

It is somewhat surprising to observe a correlation between the global unfolding around 65 °C as measured by CD (figure 8) and the local unfolding at 20 °C as deduced from the tryptic digestion (figure 4). We managed to purify a very small amount of K43T and estimated its global unfolding temperature around 60 °C from the experimental results obtained with this fraction. It is likely that global stabilization will also reduce the ease of local unfolding, but the underlying mechanisms are hard to grasp and certainly beyond the scope of this article. The observed increase in thermostability of mutant R27A has advantages for many steps along the entire line from design and purification to mass-production, pasteurisation and therefore shelf life, and real-life application in developing



C	
R17S	64.2
R17A	63.2
R27F	68.4
R27A	67.4
R45A	62.8
K64A	62.8
K86A	63.2
WT	64

Figure 8. Thermal denaturation data. A) Thermal melting curve of VHH1 obtained with CD spectroscopy (line1). Line 2 was obtained upon cooling down. B) First derivative of the heating curve in A. C) The right-hand column lists the melting temperatures (corresponding to the negative peak in the derivative curves).

countries. The next step will be animal tests with R27A to see if the *in vitro* improvements allow for rotavirus neutralization with lower doses than the wild-type.

Conclusions

We have selected a subclass of neutralizing VHHs against rotavirus that are stable under stomach conditions. To improve the application of these VHHs in the gastrointestinal tract the goal of this study was reduction of tryptic digestion of VHH1 through site-directed mutagenesis. The mutants should produce well and neutralize the rotavirus. We introduced the Frenken-accessibility plot to qualitatively predict which arginines and lysines are most susceptible to tryptic digestion. Despite its crudeness, this method worked very well in this study. It should, however, be kept in mind that this is only one study with just a few data points.

The R27A and R27F mutants both show increased resistance against trypsin and a higher melting temperature. R27A shows, like the other six mutants, affinity similar to the wild-type, which indicates that arginine 27 is not directly involved in virus binding. Surprisingly, rotavirus binding of R27F, on the other hand, is strongly reduced. Probably, this reduction is caused by steric hindrance upon virus binding caused by the phenylalanine side chain.

Kowalski (Kowalski *et al.*, 1998) saw a correlation between thermal stability of proteins and their secretion level in yeast. Our data, albeit limited in numbers, at least do not disagree with that observation.

In summary, the R27A mutant fulfils all requirements (stability, production, binding), and thus is a good candidate for further studies. For example, it still has to be shown that good *in vitro* binding correlates with good *in vivo* neutralization. Further, the trypsin concentration used in our in vitro studies is roughly similar to the concentration in the human intestines. However, the gastrointestinal tract, as every man who ever watched his production at the toilet might easily realize, contains many more compounds than pepsin, trypsin and acid. *In vivo* tests will have to shed light on these questions, but we are hopeful that this successful mutation study has generated an excellent candidate for an antibody based cure for rotavirus infections.

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Chapter 5

Comparison of VH and VHH domains on sequence level; adaptations of the VHH to the lack of VL

Edward Dolk, David Lutje Hulsik, Gert Vriend, and Theo Verrips

Abstract

Four amino acid substitutions from VH to VHH were designated as hallmarks for the single binding domain of the heavy chain antibodies (VHH). These residues occupying the VL-interface in the VH of conventional antibodies are almost always substituted in the VHH of heavy chain antibodies. These substitutions led to a more hydrophilic patch compared to the hydrophobic interaction site as seen in conventional antibodies. This clearly shows the adaptation of the VHH to its single domain status. Three seemingly unrelated studies showed that residues in this area are not only important for the solubility of the VHH, but can also be key determinants for the stability of the VHH. Here, we compare VH and VHH sequences, focusing on residues in the (former) VH-VL interface area. Furthermore we provide evidence for a role of these residues in the stability of VHHs.

1. Differences between VH and VHH

An antibody normally consists of two identical heavy chains and two identical light chains. Both chains are build up by several domains. Constant domains (C), relatively conserved domains, and variable domains (V) functioning as the binding domains of the antibody. The variable domain of the heavy and the light chain (VH and VL) form the binding unit of an antibody. These two domains are interconnected by disulphide bridges which are formed after interaction of the hydrophobic interfaces of the VH and VL.

Camelids possess next to these conventional antibodies, a subset of antibodies lacking the light chains (Hamers-Casterman, 1993). These antibodies are known as heavy chain antibodies. The variable domain of the heavy chain of these heavy chain antibodies (VHH) is therefore the single binding domain. The lack of the VL would have left a unfavourable hydrophobic patch in VHHs. However, VHHs are well adapted to their single domain status (Van der Linden, 1999; Muyldermans, 2001)

One of the hallmarks of VHHs, clearly showing this adaptation, can be seen comparing VHHs with conventional VH domains. A number of hydrophobic residues in VH domains are constitutively substituted to more hydrophilic residues in VHHs. These substitutions reside in the VL-interface of VH domains. VHHs, lacking the VL, do not

benefit from a hydrophobic interaction site and are adapted by hydrophilic substitutions in this area, which we will call the 'former' VH-VL interface.

Other mechanism showing adaptation to the single domain status have been described as well (Muyldermans, 2001). First, a longer CDR3 is often seen in the VHHs. This extention of the third binding loop can compensate for the lack of the three CDRs of the VL (Desmyter, 2001), which normally, together with the three CDRs of the VH, form the binding region. This longer CDR3 of the VHH does not only enlarge the contact surface of the antibody fragment with the antigen, it also enables a higher variety of binding motifs. In VHHs long CDR3s have been implicated to be able to protrude the active clefts of enzymes, thereby inhibiting or modulating their activity (Transue, 1998). This was thought to be difficult with conventional antibodies, which often bind their antigen in a binding cleft between VH and VL. A long CDR3 has also been implicated to be able to cover the 'former' VL-interaction site and bind the antigen similar to a VH-VL pair, only without the VL (Spinelli, 2001).

The higher variability in sequences and the use of framework residues in binding of the antigen shows adaptation to the lack of the three binding loops of the VL (Nguyen, 2000). It has been suggested that CDR1 was enlarged to cover residues 27-35 (Nguyen, 2000). Residues 27-30 in VHH show a higher variability compared to VH domains. Normally, the substitutions observed in the germline, F27Y and F29Y, would not have a major impact on the variability of the loop. However, with these mutations, the codon of Phe TTY (where Y is T or C) has been changed into TAY for Tyr. This change in codons results in the introduction of two hotspots for somatic mutation (Milstein, 1998). This could explain the higher variability of residues 27-30 in VHHs and implicates that these residues have become important in binding the antigen. Furthermore, the longer CDR1 will, like the prolonged CDR3, give more flexibility in antigen binding.

2. The 'former' VH-VL interface in VHH domains

The VH-VL interactions in conventional antibodies are limited to a number of residues. In the VH: S/H35, V37, Q39, L45, W47, F/Y91, A/L93, X95[1], F/Y100*[2] and W103. ([1] Not conserved, CDR3 residue. [2] Residue three positions before W103, in CDR3) are the ten residues normally in contact with the VL (Vargas-Madrazo, 2003). S/H35, V37, A/L93 and X95[1] have been reported to have little or no contact with the VL (Vargas-

Madrazo, 2003). This leaves Q39, L45, W47, F/Y91, F/Y100*[2] and W103 in close contact with the VL. These, all large, mostly aromatic residues form a hydrophobic plain, which enables hydrophobic interactions with the VL .

In camelid VHHs four residues, substituted from VH to VHH, V37F, G44Q/E, L45R and W47G/F/L, have been designated as hallmarks for VHHs on many occasions (Vu, 1997; Harmsen, 2000; Muyldermans, 2001). Remarkably, but not surprisingly, these substitutions are in the region where normally the VL interacts with the VH. The other residues in this area of the VHH also show some remarkable substitutions, compared to VH. Table 1 shows the residues which have been shown to interact with the VL in VHs and their distribution in 700 VHHs, currently present in our database. Residue 11 was taken into account because this residue was shown to interact with the CH1 domain in VHs (Padlan, 1994). The CH1 domain is not present in VHHs. Two residues, position 43 and position 46, which are in the interface with the VL, but not directly in interaction with the VL, were added to this table. Most striking is of course the substitution of the four amino acids already mentioned. However, there are some other noticeable substitutions, although not as conserved as the four mentioned above. Mainly, large, aromatic and hydrophobic residues are substituted for charged hydrophilic residues.

A conserved Leu at position 11 was reported to be part of a ball-and-socket connection between the VH domain and the CH1 domain (Lesk, 1988). In camelid VHHs, lacking the CH1 domain, this L11 is likely to be mutated because of the solvent exposed nature of this hydrophobic residue. Indeed, in dromedary sequences this L11 was consistently substituted into S11 (Vu, 1997), suggesting the replacement into a smaller and more hydrophilic residue, to make the VHH more soluble. However, in the llama, also lacking the CH1 domain, this substitution is not conserved. Surprisingly, 84% of the VHHs in our database, primarily llama sequences, contained a Leu at position 11. This might suggest that mutation of L11 could have structural implications in llama VHHs, which have already been overcome by dromedary VHHs. It is worthwhile investigating the importance of L11 in llama VHHs.

Position 35, although not in very tight contact with the VL, is located in the VH-VL interface. Vargas-Madrazo and co-workers showed that position 35 is predominantly occupied by Ser (40% in human VHs) or His (21% in human VHs), and in 97% of the cases orientated towards the antigen binding site (Vargas-Madrazo, 2003). In VHHs, Ala (36%)

Table 1. Distribution of residues in the (former) VH-VL interface in VHs (Vu, 1997; Vargas-Madrazo, 2003) and camelid VHHs.

Position	VH	VHH	Perc (%)	Position	VH[1]	VHH[2]	Perc (%)
11	L	L	84.1	46	E	E	91.0
		S	11.5			D	5.8
		X	4.4			X	3.3
35		A	36.0	47	W	F	36.4
		G	18.8			L	35.6
		D	7.6			G	12.0
	S	S	6.9			X	16.0
	H	H	1.9	91	Y	Y	84.1
		X	28.9		F	F	5.0
37		F	55.8			X	10.9
		Y	40.9	93	A	A	57.3
	V	V	0.6			N	23.4
		X	2.6			L	0.0
39	Q	Q	94.8			X	19.2
		X	4.2	95		R	19.8
43	K	K	85.5		D	D	16.5
		T	4.2			E	7.8
		N	4.2			K	4.2
		Q	1.7		G, Y, S	X	51.6
		X	4.4	100*		ND	
44		E	56.9	103	W	W	95.8
		Q	29.9			R	1.7
		R	2.3			E	0.3
	G	X	10.9			X	2.2
45	L	R	97.2				
		C	1.2				
		X	1.6				

and Gly (19%) are the predominant amino acids, suggesting a different role for this position in VHHs. The introduction of two hot spots for somatic mutation at position 27 and 29 can enlarge CDR1 from residue 31-35 in VHs to 27-35 in VHHs (Nguyen,

2000). This extention of CDR1 towards the N-terminus might result in a less prominent role for position 35 in antigen binding. It could be that this position becomes more important as a residue important for orientation of CDR1 and connecting the two β-sheets. The high occurrence of G35 in VHHs supports this hypothesis.

Position 35 together with position 95 were described as important for the 'proximate zone' in conventional antibodies (Vargas-Madrazo, 2003). This 'proximate zone' is defined by the residues in the interface, which are most proximate to the antigen binding. These residues are often involved in antigen binding and it is thus not surprising that less hydrophilic residues like D95 are abundant. Gly, Tyr, and Ser residues are often observed at position 95 as well, indicating that this residue could be responsible for the formation of the hydrophobic cleft often seen between VH and VL. In llama VHHs position 95 is highly variable as well, but charged, hydrophilic residues are predominant. This again shows, although less prominent, adaptation to the lack of VL.

Likewise, at position 93 a Ala is often seen in VH as well as in VHH. However, an Asn is also observed in 24% of the VHHs. Although not as prominent as the previous mutations this also makes the VHH more hydrophilic.

Two Trp residues are very prominent in the contact between VH and VL. As shown in table1 W47 is substituted in all VHHs and is considered the hallmark for camelid heavy chain antibodies. Surprisingly, W103 is still highly conserved, 95.8 % of all VHHs still have W103, although when mutated an R or E is seen in 13 out of the 27 substitutions. This indicates that the function of the two Trp residues is completely different. Substitution of W47 will be important for solubility, while W103 might have a more structural role in VHHs and could be important for correct folding or stabilisation of the inner core.

It was shown by so-called 'camelizing' of human VHs that one of these substitutions V37F has an effect on the thermostability of a human VH domain (Davies, 1996). Davies and Riechmann also suggested that mutations in FR2 could have a significant effect on stability and production levels (Davies, 1994). We obtained results that indicate an effect of mutations in FR2, residing in the 'former' VH-VL interface, on stability and production levels in a series of independent experiments.

3. Mutations to increase stability

In the first study we examined the possibility to select for stability during the phage display procedure. Phage display was performed for the selection of VHHs against a cell surface protein of *Malasezzia furfur* (Malf1) under physiological conditions (PBS). This resulted in a large pool of VHHs specific for Malf1. However, the application of these VHHs required functionality in high concentrations of shampoo. The selected VHHs were shown not able to bind to Malf1 in low amounts of shampoo. Therefore, phage display in the presence of shampoo was performed and this resulted in VHHs which were able to bind to Malf1 in shampoo, indicating a significant increase in stability of these VHHs. Investigation of the protein sequences of these VHHs revealed that residues in the 'former' VH-VL interface contained a few substitutions which do not occur very frequent in VHHs.

Residue Q/E44 is one of the four residues considered as a hallmark for the VHHs, constitutively substituted compared to the G44 present in most VHs. In the VHHs selected in shampoo R44 was found in several good binders. Mutations Q44R led to increased stability in shampoo, while R44Q decreased stability significantly (see chapter 2). Furthermore, R44K did not influence binding in shampoo. The binding in shampoo was diminished at pH 11, where K44 is less protonated. This revealed that the positive charge of R44 is responsible for the stability in shampoo.

This confirmed previous observations that charge is important in this area of the VHH. The conserved hydrophobic stretch KGLEW of VHs, is replaced by KEREF/L in VHHs. Investigation of the crystal structure of the shampoo selected VHHs did not reveal any major structural changes yet, which could explain the increased stability in shampoo. Residue T43, which also occurred in several PBS selected VHHs, was highly conserved in the shampoo selected VHHs. Closer investigation of this KEREF/L region in stability is necessary to shed light on the role of charge in this area.

4. Proteolytic stability

In a second study a mutational screen was performed to enhance the proteolytic stability of a VHH neutralizing rotavirus. VHH1 was shown to be able to inhibit rotavirus *in vitro* and *in vivo* (Van der Vaart, in preparation). To come to an economically feasible product for developing countries for decreasing the deleterious effects of rotavirus, all

features of the VHH should be optimised. In this study the goal was to decrease the susceptibility for trypsin. Mutant R27A showed increased stability against trypsin, increased thermostability, increased production levels and equal affinity compared to the wildtype.

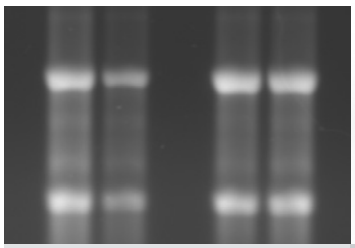
Two other mutants were made in the 'former' VH-VL interface. First mutant R45A did not show any dramatic changes. Trypsin susceptibility was not affected, affinity was not affected, production levels were slightly decreased and thermostability was slightly decreased. This indicates that despite the high conservation of R45, 97% (see table1), certain mutations may not have significant effects on stability.

Mutation of position K43, which is also highly conserved, 86%, did show dramatic effects on stability. While T43 was present in 4% of all VHHs, K43T resulted in very low production levels, due to intracellular accumulation. Thermostability of this mutant was also decreased.The intracellular fraction of the yeast cells producing this mutant did show VHH accumulation on a coomassie stained gel, however, this fraction could not be purified, probably due to aggregation. This indicates that secretion is severely hindered, which could be caused by improper folding.

5. Instability of RNA

In a third study the mutations beneficial for stability of VHHs in shampoo observed were tested on other VHHs. Selection in shampoo resulted in a number of VHHs with highly identical protein sequences and two characteristic residues, T43 and R44. However, this improved stability was not restricted to stability in shampoo, also stability in urea and guanidine-HCl was improved. In this light, VHH-H14, a VHH against the human chorionic gonadotropin hormone, and VHH-R2 against the azo-dye RR6 were mutated. Protein production levels of double mutants, with substitutions K43T, and E44R, were poor. In order to see whether low production was caused by improper folding of the protein or decreased mRNA levels, mRNA expression was determined. The mutant VHHs show a dramatic decrease in mRNA stability (figure 1).

Figure 1 shows that the mRNA expression of mutant H14M (K43T and E44R) is decreased three-fold compared to wildtype H14, and mutant R2M (K43T and E44R) more than two-fold compared to wildtype R2 mRNA levels. As a control experiment the mRNA

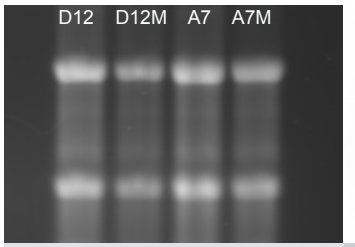


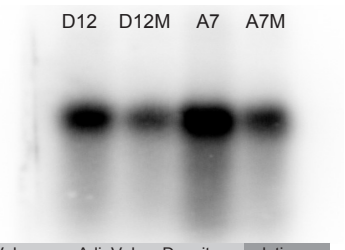
Name	Volume CNT*mm2	Adj. Vol. CNT*mm2	Density CNT/mm2	Volume CNT*mm2	Adj. Vol. CNT*mm2	Density CNT/mm2	Relative density
H14	71887.95	46296.98	255465.3	581228.5	556691.3	6703903	12.02
H14M	50506.13	24915.16	179481.6	116168.4	91631.25	1339889	3.68
R2	71524.94	45933.97	254175.3	757993.5	733456.3	8742715	15.97
R2M	68413.16	42822.19	243117.1	291051.6	266514.5	3356996	6.22
Control	25590.96	0	90941.57	24537.19	0	283012.5	
loading control				northern blot			

Figure1 Northern blot of H14, R2 and two mutants. In the left top corner the ribosomal bands as seen on the RNA gel. In the right top corner the northern blot. At the bottom the volume calculations and the relative densities.

levels of VHHs against Malf1 were determined. Four VHHs, two containing T43R44 (D12 and A7M) and two containing T43Q44 (D12M and A7) showed roughly equal amounts of mRNA (see figure 2). This indicates that the mutations made caused no dramatic instability of the mRNA.

Despite the instability of the mRNA of H14M and R2M, a small amount of mutant VHH protein could be purified and was tested for stability in shampoo. Unfortunately, the results indicated that the mutations did not result in an increased binding in shampoo of VHH-R2 and VHH-H14. This indicates that while these residues have been shown to be important for stability in shampoo, they are not the sole determinants for this stability.





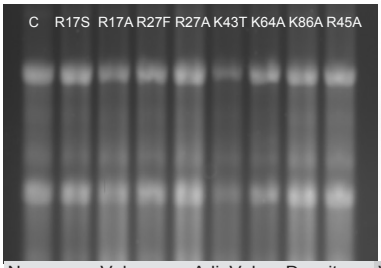
Name	Volume CNT*mm2	Adj. Vol. CNT*mm2	Density CNT/mm2	Volume CNT*mm2	Adj. Vol. CNT*mm2	Density CNT/mm2	relative densities
D12	71960.08	50884.08	246101.4	1611370	1543638	18585583	30.34
D12M	55499.76	34423.75	189807.6	1351415	1283682	15587246	37.3
A7	68045.89	46969.88	232715	2017859	1950126	23274032	41.51
A7M	57624.28	36548.27	197073.4	1335602	1267869	15404862	34.7
Control	21076	0	72079.34	67732.78	0	781231.4	
loading control				Northern blot			

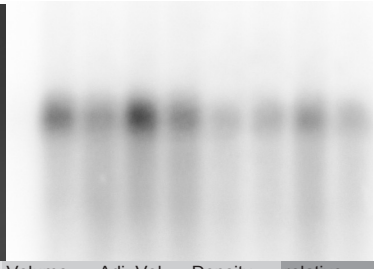
Figure2 Northern blot of D12, A7 and two mutants. In the left top corner the ribosomal bands as seen on the RNA gel. In the right top corner the northern blot. At the bottom the volume calculations and the relative densities.

As codon usage was shown to have influence on the stability of mRNA (Gouka, 1996) it could be that codon usage was not optimal for these mutants. The codons used in malf-D12 were ACC (T), CGG (R), and the codons used in H14M and R2M were ACG (T) and CGG (R). Thus, codon usage for R44 could not have solely caused the instability of the mRNA, although this codon was the least common codon for Arg (1.7 per 1000) used in *Saccharomyces cerevisiae*. Codons usage in *Saccharomyces cerevisiae* for Thr was ACT (20.2), ACA (17.7), ACC (12.6), and ACG (8.0). The codon usage for H14M and R2M was less preferable compared to codon usage of malf-D12 for production in *Saccharomyces cerevisiae*. This could be an explanation for the decreased stability of the mRNA. However, mRNA stability is complex and this might not be the only reason for the instability.

Mutant K43T of anti-rotavirus VHH1, constructed to increase proteolytic stability, also showed dramatically decreased protein production levels. To exclude that a decrease in mRNA stability caused the decreased production, as seen in the mutants of H14 and R2, levels of mRNA expression were determined. Figure 3 shows the Northern blot of the eight mutants produced for the mutational screen on decreasing trypsin susceptibility.

Figure 3 shows there is no significant decrease of mRNA of mutant K43T. This indicates that the hampered secretion was due to a problem on protein level and further supports the hypothesis that this mutation causes a folding problem.





Name	Volume CNT*mm2	Adj. Vol. CNT*mm2	Density CNT/mm2	Volume CNT*mm2	Adj. Vol. CNT*mm2	Density CNT/mm2	relative density
control	49143.95	23135.03	176269.5	NA	NA	NA	
R17S	55850.76	29841.84	200325.5	293287.7	263675.7	4545334	11.4
R17A	53846.4	27837.48	193136.3	261696.4	232084.4	4055737	7.78
R27F	48245.27	22236.35	173046.2	399910.9	370298.9	6197766	13.3
R27A	37726.85	11717.93	135318.7	291666.2	262054.2	4520204	11.78
K43T	55411.94	29403.02	198751.6	174869.6	145257.6	2710105	12.4
K64A	48851.53	22842.61	175220.7	181309	151697.1	2809904	5.16
K86A	45084.04	19075.12	161707.5	238131.8	208519.8	3690535	9.13
R45A	52131.41	26122.49	186985	161803.1	132191.1	2507604	6.93
empty	26008.92	0	93288.81	29611.99	0	458922.8	

Figure 3. Northern blot of mutants of VHH1. In the left top corner the ribosomal bands as seen on the RNA gel. In the right top corner the northern blot. At the bottom the volume calculations and the relative densities.

Conclusions

Residues in the VH-VL interface are highly conserved in conventional antibodies. These residues are usually large, hydrophobic and aromatic residues in the VH, with the specific function of interacting with the VL and formation of the binding cleft. Without any further practical knowledge one could easily assume that these residues in VHHs, lacking the VL, are less important and will therefore be less conserved. In this respect, it is clear that these residues in VHHs have a higher variability. Nevertheless, the importance of these residues is shown by the unique properties obtained by substitution of the hydrophobic residues into more hydrophylic and often charged residues. High solubility and thereby high production levels in microorganism is just one example (Frenken, 2000).

A peculiar exception to these substitutions from hydrophobic to hydrophylic residues is W103. This large aromatic and very hydrophobic residue is seen in 96% of all VHHs as well. This implicates a different role for W103 in VHHs compared to the other residues in the 'former' VH-VL interface.

The substitutions observed from VH to VHH clearly increase the hydrophilicity. Here, we provided evidence for a broader function of the residues in the 'former' VH-VL interface. We claim that these residues, especially the residues in the hydrophilic KEREF/L stretch are key determinants for the stability of VHHs. Investigation of the residue distribution showed the highest rate of substitutions in this area. G44Q/E (87%), L45R (97%), and W47F/L/G (84%). Furthermore, mutations of position 43, 44 and (to a lesser extent) 45 had dramatic effects on stability. This suggests that these residues have a key role in the stability of the VHHs.

Material and methods

Distribution of residues in VHH

A MySQL database containing 703 public and proprietary camelid VHH sequences, primarily llama sequences, was queried based on residue and kabat number. The occurance of each residue was determined for each position.

Northern blot

Yeast cells were disturbed with 0.45 mm glass beads in phenol and RNA extraction buffer (50 mM Tris pH 7.4; 100 mM NaCl; 10 mM EDTA) in a Bead Beater (Biospecs Products Inc.) to isolate total RNA. A phenol/chloroform extraction was performed, and subsequently total RNA was precipitated by adding 3 M NaAc pH 5.6 and ethanol. RNA was washed with ethanol, air-dried and resuspended in ddH2O. Ten μg of total RNA with 2 μl ethidiumbromide was loaded on a 1% denaturing formamide/ formaldehyde gel. Total RNA was separated by electrophoresis and ribosomal bands were visualised with an Imago gel imaging system (Isogen Life Science, Maarssen, The Netherlands). RNA was transferred to Hybond-N membrane (Amersham Pharmacia Biotech) and cross-linked using a UV-stratalinker (Statagene). Probes against VHH-H14, VHH-R2, anti-Malf VHHs and anti rotavirus VHHs was obtained by PCR. 25 ng of purified PCR product was used for incorporation of 50 μCi ^{32}P-CTP (Amersham) using the Prime-a-Gene labelling system (Promega) according to the manufacturer's protocol. Unincorporated ^{32}P-dCTP was removed using NICK columns (Pharmacia) according to the manufacturer's instructions. The blots were incubated for prehybridisation in hybridisation mixture (1mM EDTA; 7% SDS; 0.5 M $NaPO_4$ pH 7.5) for one hour in a micro-4 hybridisation oven (Biozym) at 45 °C. Probes were added and hybridised overnight at 45 °C. Subsequently, blots were washed once in 2X SSC at room temperature for two minutes, twice in 2X SSC, 0.1% SDS, 0.1% NaPPi for 20 minutes at 45 °C, and once for 20 minutes in 0.5X SSC, 0.1% SDS, 0.1% NaPPi at 45 °C. After a final wash with 2X SSC at room temperature, the membrane was wrapped in Saran (Dow Chemicals), and autoradiograms were developed using hyperfilm MP (Amersham). To quantify the RNA levels, blots were exposed for autoradiography and signals quantified using a BioRad Molecular Imager FX (BioRad, Veenendaal, the Netherlands). Volume calculations were performed using Quantity One software (Bio-Rad, Veenendaal, the Netherlands). Relative densities were determined using the volumes of the ribosomal bands as a control. *S. cerevisiae* codon usage was obtained from http://www.chem.ucla.edu/~pdoucett/icosa/Yeastcode.html.

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Chapter 6

General discussion

General discussion

In this thesis the stability of llama heavy chain antibody fragments, VHHs, was investigated under several extreme conditions. Survival of these conditions or functionality under these conditions can be necessary for the application of VHHs in consumer goods, process aids and research tools. The applications in which antibodies are used are numerous and still increasing. Every application has its specific demands concerning the stability of the antibodies, either in the application itself or in the line of process. The VHH, being the smallest naturally occurring binding unit, is already privileged with many beneficial properties compared to conventional antibodies.

Temperature stability

VHHs have unique characteristics at high temperatures. They were already shown to be functional after a heat shock of two hours at 90°C (Van der Linden, 1999) and were able to refold after heat denaturation (Perez, 2001). These unique properties of VHHs are likely to be direct benefits from the single domain status. Chapter two describes that VHHs are able to refold to their native conformation even at 80°C, induced by addition of the antigen. They were able to form a stable antibody-antigen complex at high temperature. The only condition for the refolding at these high temperatures is a high affinity binding under normal conditions. The high stability of the antigen-antibody complex is one reason for the fast and complete refolding of the VHH population. The other reason for this phenomenon is most likely refolding of the temperature denatured antibody directly induced by binding of the antigen to the unfolded VHH, as described in chapter 2. An interaction between a peptide comprising CDR3 and the antigen was observed. This observation together with the remarkable refolding at high temperatures could indicate that there is an interaction between CDR3 in the unfolded VHH and the antigen, which could be a catalyst for the formation of the native antibody-antigen complex. Long range interactions within the VHH necessary for correct folding are more likely to occur, caused by stabilisation through binding of the antigen.

The refolding capacity, even under extreme conditions, also implies that the functionality of VHHs only decreases when irreversible denaturation caused by

aggregation, proteolysis or chemical degradation has occurred. Heat denaturation, caused by pasteurisation or sterilisation of food products containing antibodies, can be deleterious for conventional antibodies. VHHs are able to survive these harsh conditions and this is a great advantage when using VHHs in applications where these heat shocks are required. The first signs are already emerging that this mechanism also holds true for stability in other denaturing conditions and is not reserved to only reversible temperature unfolding.

Selection and screening

Several advantages of VHHs, like the good solubility and the refolding capacity, are intrinsic properties of VHHs. On the other hand, other advantages are abilities of a small population of VHHs, either already present or easily incorporatable. When present it is necessary to select for this small population of VHHs, in order to find the VHH with the desired properties. Van der Linden showed that in a population of VHHs binding to a dye RR6, with relatively high sequence similarity, only two VHHs were able to bind RR6 at 90°C (Van der Linden, 1999). If functionality at 90°C is required, like in laundry products, selection or screening for these properties will be necessary. In chapter 3 a successful method is described in order to select for beneficial properties of VHHs. Applied phage display has been shown to be able to efficiently select for stability in a desired environment, in this case high concentrations of shampoo.

Phage display with VHHs is a unique combination. *In vivo* maturation of the antibody fragments by immunisation is combined with easy cloning. For this reason highly diverse libraries can easily be constructed for almost any antigen. Several tricks, like elution with specific domains or competitors to select for specific epitopes, decreasing antigen concentration for the selection towards high affinity binders or the use of counter-selections for ridding binders on unwanted epitopes, make the selection procedure very flexible. The flexibility in the selection allows for efficient and large-scale selection of the desired VHH in a relatively short time. Therefore subsequent laborious protein engineering can be minimised

Phage can be the rate limiting step in the phage display procedure. It is possible that the VHH survive the extreme condition applied during the selection procedure and are able to bind to their antigen, but because of disruption of the phage, the phage-VHH couple is no longer able to reinfect bacteria after selection. In this scenario one could miss efficient

binders. There was no loss of infectivity observed for the selection in shampoo, the non- and anionic surfactants in shampoo could be considered as an extreme condition. On the other hand the addition of pepsin during the selection procedure for rotavirus inhibiting VHHs showed a loss of recovered phage after selection. This may have been caused by digestion of the protease sensitive link between VHH and phage (Van der Vaart, manuscript in preparation). On the other hand, a large population of phage-VHH combinations were still present. Specific binders and inhibitors of rotavirus could be obtained, which are stable in the environment of the gut, including pepsin.

Protein engineering

One way of incorporating beneficial properties into VHHs is by protein engineering. DNA shuffling (Van der Linden, 2000), random mutagenesis (der Maur, 2002) or grafting CDR loops on stable frameworks or other scaffolds (Jung, 1997) are methods used for improvement of antibodies. Site-directed mutagenesis requires structural knowledge from previous experiments and observations (Davies, 1996).

The high homology of the VHH domains and the rising structural knowledge of these binding blocks make protein engineering an appreciated tool for the improvement of VHHs. However, it has been shown that beneficial mutation of a residue in one VHH does not necessarily have to be beneficial to the other. Chapter four describes the improvement of a VHH by a mutational analysis. In this study we inserted a mutation which was present in a subpopulation of VHHs stable in shampoo and urea to enhance the properties of this VHH. However, where mutation K43T was beneficial for VHHs binding Malf1 in shampoo, it was deleterious for the secretion and probably the folding of VHH1 binding rotavirus.

Mutant R27A was found to be a good candidate to become an economically feasible product in developing countries for the reduction of the incidences of rotavirus infection. This mutant was obtained in a relatively short time and its properties are already excellent for the use in the gastrointestinal tract. First, a pool of VHHs was selected via phage display in the presence of a low pH and pepsin. This reinsured the stability of the VHHs in the environment of the gut. Subsequent screening on inhibition of rotavirus *in vitro* resulted in a population of VHHs able to inhibit rotavirus. In order to be functional in the intestine trypsin stability was improved via protein engineering.

Homology modelling and the introduction of the Frenken-accessibility plot to qualitatively predict which arginines and lysines are most susceptible to tryptic digestion, resulted in a small group of possible beneficial mutants (Dolk, submitted for publication). This method proved to be very efficient, the mutant which was most likely to improve the properties of the VHH was shown to be the first cleavage site for trypsin. As shown before the stability against trypsin cleavage increased dramatically after removal of this first cleavage site. The Frenken-accessibility plot could therefore be a very efficient tool to predict the first cleavage site of serine-proteases in proteins.

We still do not know what the boundaries are to the applications of the VHHs. They can be reused on a affinity chromatography column for over 2000 times without loss of capacity (Verheesen, 2003). They can be used under denaturing conditions often used in biochemical studies, can be coupled easily and site specific, and are therefore excellently suitable for protein arrays (Zhu, 2000; Haab, 2003). They can be used to combat bacteriophage infections in dairy plants (Ledeboer, 2002; de Haard, accepted for publication). The uniqueness lies in the flexible selection procedure and the ability to select that one binder which has all the properties needed for your application.

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Summary

Camelids have next to their normal antibodies, a unique subset of antibodies lacking light chains. The resulting single binding domain, VHH, of these heavy chain antibodies consequently have unique properties. A high stability is one of these properties, which was investigated in this thesis.

The applications in which these VHHs are to be used, require functionality in non-physiological environments. High temperature, anionic and non-ionic surfactants in shampoo, and the low pH and digestive enzymes of the gastrointestinal tract put high demands on the stability of the VHHs.

Regardless of these harsh conditions, the VHHs seem to exceed conventional antibody fragments and are known for their high stability. Stability in the previous sentence must be regarded as functionality under these conditions. In chapter 2 we showed that VHHs can be functional at high temperatures, despite of the fact that they were already unfolded at high temperatures. Unfolding of the VHH population started at 60 ºC, and most of the population of VHHs is unfolded at 80 ºC. However, after addition of the antigen, complete refolding and complex formation was observed. This shows the fast and highly efficient refolding of the VHHs, which can be of use in a wide range of applications.

The single domain of the VHHs is the logical cause of the ability of refolding and explains why refolding is not observed in more complex antibodies, being composed of several domains, which are not able to refold properly. This refolding property of VHHs, together with the low degree of aggregation, implies that these VHHs can be functional beyond their intrinsic stability.

The adaptations to the single domain status shown by VHHs, reside predominantly in the region where in conventional antibodies the VL is attached. Four substitutions are hallmarks for VHHs, making the 'former' VL interface highly hydrophilic. Furthermore, chapter five describes that more substitutions can be observed from VH to VHH. Most of these substitutions are from large hydrophobic residue to hydrophilic (charged) residues. These substitutions are not only involved in making the VHH more soluble, in chapter three and four two mutational studies are described which indicate that the residues in this so-called 'former' VH-VL interface could also have a prominent role in the stability of the VHHs.

Chapter three describes the easy and rapid selection of VHHs able to bind in shampoo by addition of shampoo to the selection protocol. The VHHs obtained from this selection were investigated and the protein sequences show that selection is driven towards a small subclass of VHHs. This subclass exceed other VHHs for stability in shampoo, indicating that selection was directed towards stability in shampoo.

This technique was used in a second study to ensure stability of VHHs in the environment of the gut. VHHs against rotavirus were selected at low pH and in the presence of pepsin. To further enhance the stability of the selected subclass of VHHs, protein engineering was used to ensure stability in the intestine. A mutational study was performed to remove trypsin cleavage sites from the VHH. Mutant R27A showed a reduced trypsin susceptibility. Furthermore, this VHH also showed increased production levels, increased thermostability and equal affinity compard to the wildtype VHH1.

These results show that VHHs are ideal candidates for a wide range of applications. As shown in this thesis, the high stability of these VHHs, together with the rapid and easy selection methods, and subsequent improvement by protein engineering, results in exquisite opportunities for application in harsh conditions.

Samenvatting in het Nederlands

Het immuunsysteem heeft de taak om ons lichaam te beschermen tegen pathogenen, zoals virussen en bacteriën. Antilichamen hebben een belangrijke taak in dit proces. Ze herkennen de niet-lichaamseigen stoffen (antigenen), binden aan deze antigenen en zorgen hiermee voor de vernietiging van de antigenen via een cascade van gebeurtenissen. De primaire eigenschap van antilichamen is het specifiek binden van de antigenen met een hoge affiniteit. Deze eigenschap maakt antilichamen bruikbaar in toepassingen buiten het lichaam, in zowel de industrie als de wetenschap. In dit proefschrift staan voorbeelden beschreven voor de toepassing van antilichamen in wasmiddelen, voor het voorkomen van roos en voor de inhibitie van rotavirus infectie.

Voor de toepassing van antilichamen in producten is het van belang dat deze antilichamen functioneel zijn buiten hun "normale" omgeving, de fysiologische omstandigheden van het bloed. Hoge temperaturen nodig voor sterilisatie of pasteurisatie, hoge concentraties zeep in shampoo of de zure omgeving van het maagdarmkanaal met alle aanwezige stofwisselingsenzymen mogen aangemerkt worden als extreme omstandigheden voor antilichamen.

Antilichamen zijn complexe eiwitten die opgebouwd zijn uit verschillende eiwitdomeinen (zie figuur 1 van de introductie). Twee van deze domeinen, de variabele domeinen vormen samen het bindende deel van het antilichaam. Deze domeinen bestaan uit strengen van aminozuren die op een zeer specifieke manier gevouwen worden waardoor er drie loops per domein ontstaan die samen zorgen voor de specifieke binding van het antilichaam aan het antigen. Normale antilichamen zullen extreme omstandigheden niet kunnen weerstaan omdat de specifieke vouwing van onder andere deze variabele eiwitdomeinen verloren gaat. Door de ontvouwing zullen de domeinen makkelijk kunnen aggregeren en niet meer terugvouwen naar hun oorspronkelijke conformatie. Hierdoor verliest het antilichaam zijn bindende capaciteit en is niet meer functioneel.

Lama's, kamelen en dromedarissen bezitten naast deze "normale" antilichamen een bijzondere klasse antilichamen. Deze zware keten antilichamen missen de zogenoemde lichte keten, wat tot gevolg heeft dat het bindende deel van deze antilichamen bestaat uit slechts één domein. Er is aangetoond dat dit domein, VHH, zich aangepast heeft aan het ontbreken van het tweede bindende domein. In hoofdstuk vijf staan een aantal van deze

aanpassingen beschreven. Op sequentieniveau is gekeken naar de verschillen tussen normale en lama antilichamen. De conclusie is dat hydrofobe aminozuren vervangen zijn door hydrofiele aminozuren om aggregatie te voorkomen. Met name de regio waar normaal het tweede domein geassocieerd is laat deze aanpassingen zien. Daarnaast zorgt een langere derde bindingsloop van de VHH's voor unieke vouwings-mogelijkheden, welke ook compenseren voor het gebrek van het tweede bindingsdomein. Dit maakt de VHH gelijkwaardig aan normale antilichamen op functioneel gebied.

In hoofdstuk twee, drie en vier staan voorbeelden beschreven van de toepassing van deze antilichamen onder extreme omstandigheden. In hoofdstuk twee is gekeken naar de stabiliteit van deze antilichamen bij hoge temperaturen. VHH's zijn ontvouwen bij 80 ˚C, maar zijn in staat om terug te vouwen door de aanwezigheid van het antigen. Het ontvouwen antilichaam kan een interactie aangaan met het antigen, waardoor hervouwing bij 80 ˚C efficiënt kan verlopen.

In hoofdstuk drie staat een selectiemethode beschreven die gebruikt kan worden om VHH's te selecteren voor de toepassing onder extreme omstandigheden. Aangetoond is dat selectie met faag display in de aanwezigheid van shampoo zorgt voor selectie van VHH's met hogere stabiliteit in shampoo.

In hoofdstuk vier staat de optimalisatie van een VHH voor het gebruik in het maagdarmkanaal beschreven. Een VHH tegen rotavirus is geselecteerd op stabiliteit in zuur milieu en pepsine (maag), volgens de methode beschreven in hoofdstuk drie. Hierna is de stabiliteit tegen trypsine (darmen) verbeterd door gerichte mutaties aan te brengen. De unieke combinatie van deze technieken heeft geleidt tot een VHH met verbeterde eigenschappen qua trypsine stabiliteit, productie, temperatuur stabiliteit en gelijke affiniteit. Dit maakt deze mutant een goede kandidaat voor een economisch rendabel product voor de remming van rotavirus infectie, zelfs voor toepassing in derde wereld landen.

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Curriculum vitae

Edward Dolk werd op 30 september 1976 geboren in Vlaardingen. In 1994 behaalde hij zijn V.W.O. diploma aan het G.S.G. Helinium in Hellevoetsluis. In September 1994 begon hij aan de opleiding Biologie aan de Universiteit Utrecht. Na een eerste stageonderzoek aan de voorhersenontwikkeling van zebravissen bij het Hubrecht laboratorium onder leiding van Jos Joore en Dana Zivkovic en een tweede stageonderzoek aan het effect van signaal sequenties op de expressie van heterologe eiwitten in gist onder leiding van John Chapman en Anne-Marie Verbiest bij Unilever Research Vlaardingen, rondde hij zijn studie af in de richting Fundamentele Biomedische Wetenschappen in 1999. In September 1999 begon hij aan het promotie onderzoek dat beschreven staat in dit proefschrift onder leiding van Prof. Dr. Ir. C.T. Verrips. De uitvoering vond tussen September 1999 en 2001 plaats bij Unilever Research Vlaardingen en tussen 2001 en maart 2004 plaats bij de afdeling Moleculaire Celbiologie van de Universiteit Utrecht.