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# 'Camelising' human antibody fragments: NMR studies on VH domains

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**Abstract**

A human heavy chain variable domain (VH) was expressed in bacteria for structural analysis by NMR spectroscopy. NMR analysis was initially impossible due to the short transverse proton relaxation time of the VH, probably caused by aggregation through the exposed interface naturally in contact with the light chain. The relaxation time was improved to normal values when this interface was mutated to mimic heavy chains of camel antibodies naturally devoid of light chains and through the use of the detergent CHAPS. Assignment of NMR signals will now be possible after isotopic labeling. Implications for the design of VH domains as minimum size immunoreagents are outlined.

*Key words:* Antibody; Fv; VH; Nuclear magnetic resonance; Detergent; Camel

## 1. Introduction

Through recombinant DNA technology antibody genes can be manipulated at will. They have been mutated, dissected into domains, fused to other proteins and successfully expressed in eucaryotic cells and bacteria [1]. Many attempts have been made to design minimum size, antigen binding immunoglobulin fragments (minimum recognition units), because these might, for example, improve tissue penetration when used as immunoreagents in pharmaceutical applications. The so called Fv fragment, formed by the variable domains of heavy (VH) and light chain (VL), was most commonly viewed as this unit. However, earlier observations indicated that some heavy chains could bind antigen in the absence of a light chain [2–4]. Also VH domains with high affinities were detected in expression libraries of mouse VH genes [5]. However, a more detailed biochemical analysis of isolated VH's was hampered by their low expression and poor solubility (L.R., unpublished). More recently, camelid immunoglobulins devoid of light chains have been described [6]. The VH domains in the camelid antibodies presumably form two antigen binding sites just like immunoglobulins with light chains.

Through antibody engineering it also became possible to undertake detailed NMR studies on immunoglobulins. As triple resonance NMR techniques [7] should enable complete assignments of proteins up to 30 kDa, solution structures even of entire Fv fragments (usually 12.5 kDa VH plus 11.5 kDa VL) will eventually be obtained based on nuclear Overhauser enhancement data. Fv's were successfully labeled with $^{15}N$ and $^{13}C$ [8–11], but, to date, poor solubility and short $^{1}H$ relaxation times have prevented their complete assignment. On the other hand, an isolated VL was completely assigned [10,11]. Little progress, however, has been made with respect to isolated VH's, which would be more likely candidates for minimum recognition units.

Therefore we started the analysis of a human VH with the aim of developing a small immunoreagent and analysing it by NMR spectroscopy.

## 2. Experimental

### 2.1. DNA technology

The Ox13-VH gene was derived from the scFv clone αOx13 [12]. The VH and its mutants were expressed in the absence of a VL using a pUC19-based expression vector described previously [13]. The Ox13-VH was mutated to VH-P1 by site-directed mutagenesis in M13 with the oligonucleotide, CAMEL2 (Fig. 1). A library of VH mutants was prepared in a polymerase chain reaction and cloned into the expression vector using the newly introduced *PstI* site in CDR2 (Fig. 1) and the polylinker *HindIII* site. PCR was performed for 30 cycles at 92°C for 1 min, 36°C for 1.5 min and 72°C for 2 min with the oligonuleotide, CAMEL3 (Fig. 1) and the M13 reverse primer for amplification of the VH-P1 gene.

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*Abbreviations:* BSA, bovine serum albumin; CDR, complementarity determining region; CHAPS, 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate; 2D-TOCSY, two dimensional total correlation spectroscopy; FR, framework region; IPTG, isopropyl β-D-thiogalactopyranoside; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; PCR, polymerase chain reaction; scFv, single chain Fv fragment; VH, heavy chain variable region; VL, light chain variable region. Mutants are denoted by the original amino acid, followed by residue number and new amino acid.


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*2.2. Screening*

The VH library in the *Escherichia coli* strain, JM109, was screened for expression after transfer of bacterial colonies from TYE agarose plates containing 100 mg/l ampicillin and 1% glucose to Hybond-N membranes (Amersham). The colonies were grown on the membranes for another 6 h under identical conditions. Membranes were then washed on filter paper soaked in 2 × TY medium containing 100 mg/l ampicillin and 1 mM IPTG, placed onto several layers of nitrocellulose membranes (Schleicher & Schüll) and transferred to a new TYE agarose plate containing ampicillin and IPTG. After 24 h induction at 37°C, membranes were washed in PBS, blocked with 3% BSA in PBS and incubated with protein A conjugated peroxidase (Sigma; 1 μg/ml in 3% BSA/PBS) for 1 h at room temperature. Membranes were finally washed with PBS and developed with 4-chloro-1-naphtol/$H_2O_2$. Colonies giving the darkest spots were rescued and their double stranded plasmid DNA was sequenced by the dideoxy method.

*2.3 Protein purification*

VH was purified from supernatants of bacterial cultures grown to confluency at 37°C in 2 × TY medium containing 0.1% glucose and 100 mg/l ampicillin and after induction with 1 mM IPTG for 24 h at 30°C. Supernatants were passed through protein A–Sepharose (Pharmacia), which was then washed with 0.5 M NaCl in PBS. VH was eluted with 10 mM phosphate buffer (pH 2.6) and immediately neutralised.

*2.4 NMR spectroscopy*

$D_2O$-NMR spectra were recorded with 1 mM VH samples in 10 mM phosphate, 200 mM NaCl, $pH_{app}$ 6.2 in 99.9% $D_2O$ or in the additional presence of 10 mM CHAPS. The $H_2O$ spectrum was recorded in the presence of CHAPS under identical conditions except for the use of 90% $H_2O$/10% $D_2O$ instead of 99.9% $D_2O$. $D_2O$-TOCSY [14] ($\tau_m$ = 37 ms) spectra were acquired at 313 K on a Bruker AMX-500 spectrometer using a spectral width of 12,500 Hz (2048 complex points acquisition, 160 scans) in f2 and 240 increments for a spectral width of 4,000 Hz in f1. For the $H_2O$-TOCSY experiment ($\tau_m$ = 57 ms) 256 transients were acquired with 300 increments for a spectral width of 6,000 Hz in f1. Data were analysed with UXNMR (Bruker) software. Transverse proton relaxation times ($T_2$) were estimated from the line width (at half peak height) of the non-exchanged amide signals in $D_2O$ spectra processed in f2 without window functions. An average $J_{NHC^{\alpha}H}$ coupling constant of 8 Hz was assumed as these amides are likely to be part of the immunoglobulin domain β-sheet structure.

## 3. Results

*3.1. The human VH3 domain*

As a first step towards the design of a minimum size, antigen binding immunoglobulin fragment and its structural analysis by NMR spectroscopy, we decided to study the human heavy chain variable domain, Ox13-VH (Fig. 1), which could later also be used as a building block in the NMR analysis of a human antibody Fv fragment. The Ox13-VH was derived from the single chain Fv (scFv) αOx13 [12] and originated from the human VH3 family [15], which binds protein A [16]. This enabled a simple one step purification of both VH and scFv with protein A–Sepharose. The αOx13 scFv was selected from a library of phage surface-expressed human scFv's with randomised VH-CDR3 residues through binding to 2-phenyloxazolone [12]. The Ox13-VH on its own has no known specificity.

The Ox13-VH was expressed well even when not part of a scFv. About 6 mg of VH could be purified from 1 l of bacterial culture. In contrast VH domains of other antibody Fv fragments studied in our laboratory were expressed at very low levels (about 0.1 mg/l; L.R., unpublished). However NMR analysis of the VH (12.5 kDa) seemed impossible due to its large line width. Even at 313 K the transverse proton relaxation time, $T_2$, was estimated as only 14.5 ms for its $D_2O$ stable amides compared to 23 ms measured for the αOx13 scFv (J.D. and L.R., in preparation). The $T_2$ value is a good indicator of the success of multi-dimensional NMR experiments with larger proteins, where cross-peak heights scale proportionally to the inverse correlation time and thus the proton $T_2$ [17]. Consequently for the Ox13-VH very few cross-peaks were observed in 2D-TOCSY spectra (Fig. 2).

*3.2. 'Camelising' the human VH3*

As the broad lines of the Ox13-VH were probably due to aggregation caused by the exposed VH/VL interface, we mutated this interface through protein engineering. The modifications were based on sequences published for two VH genes found in camelid immunoglobulins devoid of light chains [6]. The sequence of their FR's and that of the human VH3 family were remarkably similar except for three residues (44, 45 and 47) in FR2 (Fig. 1), usually highly conserved in VH domains. These residues (G44, L45 and W47 in the human VH3) are located in the VH/VL interface and the side chains of residues 45 and 47 point towards the VL in known antibody structures [18].

Initially the mutant VH-P1 was engineered to match the camel VH most hydrophilic with respect to these three residues (VH-G44E, L45R, W47G). At the same time a restriction site close to these residues (causing a conservative Ile/Val mutation of residue 51 in CDR2, Fig. 1) was introduced to simplify further mutations in this region through the use of PCR. As the two published camel VH's differed at position VH-47 (glycine or phenylalanine) we also prepared a library of VH's randomised at position 47 containing Ox13-VH mutants of the type: G44E, L45R, W47X, I51V (Fig. 1).

As the engineered VH domains were to be analysed by NMR spectroscopy, highly expressed mutants seemed initially of greatest interest. Therefore the library of VH mutants was screened for expression in a blot assay of bacterial colonies using protein A-conjugated peroxidase. Sequences of the strongest-expressing clones indicated that a branched, aliphatic side chain of residue VH-47 was critical for good expression. Among nine sequenced clones three leucine, isoleucine and valine residues each were found at position VH-47. The originally designed mutant, VH-P1, gave only a weak signal in the screening experiments, whereas the wild-type Ox13-VH was clearly better expressed than even the best expressers of the library.

Two mutants were purified and analysed by NMR: the highly expressed VH-P8 (Ox13-VH-G44E,L45R,W47I, I51V) and the moderately expressed VH-P1 (Ox13-VH-

**a) Partial DNA sequence of the Ox13-VH**

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                                                        PstI
Mutants  ...      P   G   K   E   R   E  G/X  V   S   A   V   S   G
CAMEL3                   3'- CTC TCC CTC NNS CAG AGA CGT CAA TCA CCA - 5'
CAMEL2             3'- CC TTC CTC TCC CTC CCC CAG AGA CGT CAA TCA CCA T- 5'
Ox13VH 5'...GCT CCA GGG AAG GGG CTG GAG TGG GTC TCA GCT ATT AGT GGT AGT... 3'
FR2       ... A   P   G   K   G   L   E   W   V   S   A   I   S   G   S ... CDR2
             40                44  45      47          50      52  52a 53
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**b) VH protein sequences**

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FR1          1       5        10        15        20        25        30
Hu Ox13VH    Q V Q L V E S G G G L V Q P G G S L R L S C A A S G F T F S
Hu VH-P8     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hu VH-P1     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Camel VHa                    . . S . . G . . . . . . . . . I . S
Camel VHb                    . . S . . A . . . . . . . . . S . S

CDR1/FR2     31      35    36      40        45      49
Hu Ox13VH     S Y A M S     W V R Q A P G K G L E W V S
Hu VH-P8      . . . . .     . . . . . . . . E R . I . .
Hu VH-P1      . . . . .     . . . . . . . . E R . G . .
Camel VHa                   . F . E G . . . E R . G I A
Camel VHb                   . Y . . . . . . E R . F . .

CDR2         50  52 a53  55        60        65
HU OX13VH     A I S G S G G S T Y Y A D S V K G
Hu VH-P8      . V . . . . . . . . . . . . . . .
Hu VH-P1      . V . . . . . . . . . . . . . . .
Camel VHa
Camel VHb

FR3          66      70        75        80  82 a b c83  85        90      94
Hu Ox13VH     R F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A R
Hu VH-P8      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hu VH-P1      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Camel VHa     . . . . . Q . S T L K . M . . L . . N . K P . . . G T . . . . A
Camel VHb     . . . . . Q . S A . . . V . . . . . . . K P . . . . M . . . K I

CDR3/FR4     95       100 102   103 105       110   113
Hu Ox13VH     L K K Y A F D Y     W G Q G T L V T V S S
Hu VH-P8      . . . . . . . .     . . . . . . . . . .
Hu VH-P1      . . . . . . . .     . . . . . . . . . .
Camel VHa                         . . . . . Q . . . . .
Camel VHb                         . . . . . Q . . . . .
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Fig. 1. VH sequences. Protein residues are numbered according Kabat et al. [23]. Dots indicate sequence identity to the Ox13-VH. Empty spaces denote regions of the camel VH domains, for which no sequence was published [6]. S = C or G, X = any amino acid.

G44E,L45R,W47G,I51V). For VH-P8, 4 mg of protein were purified from 1 l of bacterial culture, whereas for VH-P1 it was only 1 mg.

In the NMR experiments the line width of VH-P8 was still poor ($T_2$ = 16 ms), and only few cross-peaks were observed in the NMR spectrum (Fig. 2). However, for VH-P1 a dramatic increase in the relaxation time was observed ($T_2$ = 29 ms), which now was typical for a protein of its size. Accordingly strong cross-peaks were observed in its 2D-TOCSY spectrum (Fig. 2).

Through the replacement of hydrophobic with hydrophilic residues in the exposed VH/VL interface of the Ox13-VH its relaxation time was increased from 14 ms to 29 ms. Unfortunately these mutations decreased the expression, considerably hampering for economic reasons future NMR analyses, which depend on isotopic labeling. For this reason we explored a different way to decrease the line width of the VH.

### 3.3. *Adding detergent*

Recently the detergent CHAPS was successfully used to improve the $T_2$ of the 19 kDa protein, calcineurin B [19]. The presence of even non-deuterated detergent would ultimately not interfere with the NMR analysis so long as heteronuclear techniques were applied using isotopically enriched protein.

10 mM CHAPS was added to the $D_2O$ samples of the three VH domains. The $T_2$ of the wild-type Ox13-VH increased from 14.5 ms to 20 ms and the $T_2$ of the VH-P8 mutant from 16 ms to 29 ms. The latter matched the value measured for the VH-P1 mutant in the absence of CHAPS. Now strong cross-peaks were observed for the Ox13-VH and VH-P8 in 2D-NMR spectra similar to those seen with the VH-P1 mutant (Fig. 2). In contrast addition of CHAPS to VH-P1 improved neither its line width nor the TOCSY spectra (Fig. 2).

The effect of the detergent seemed to be highly specific.

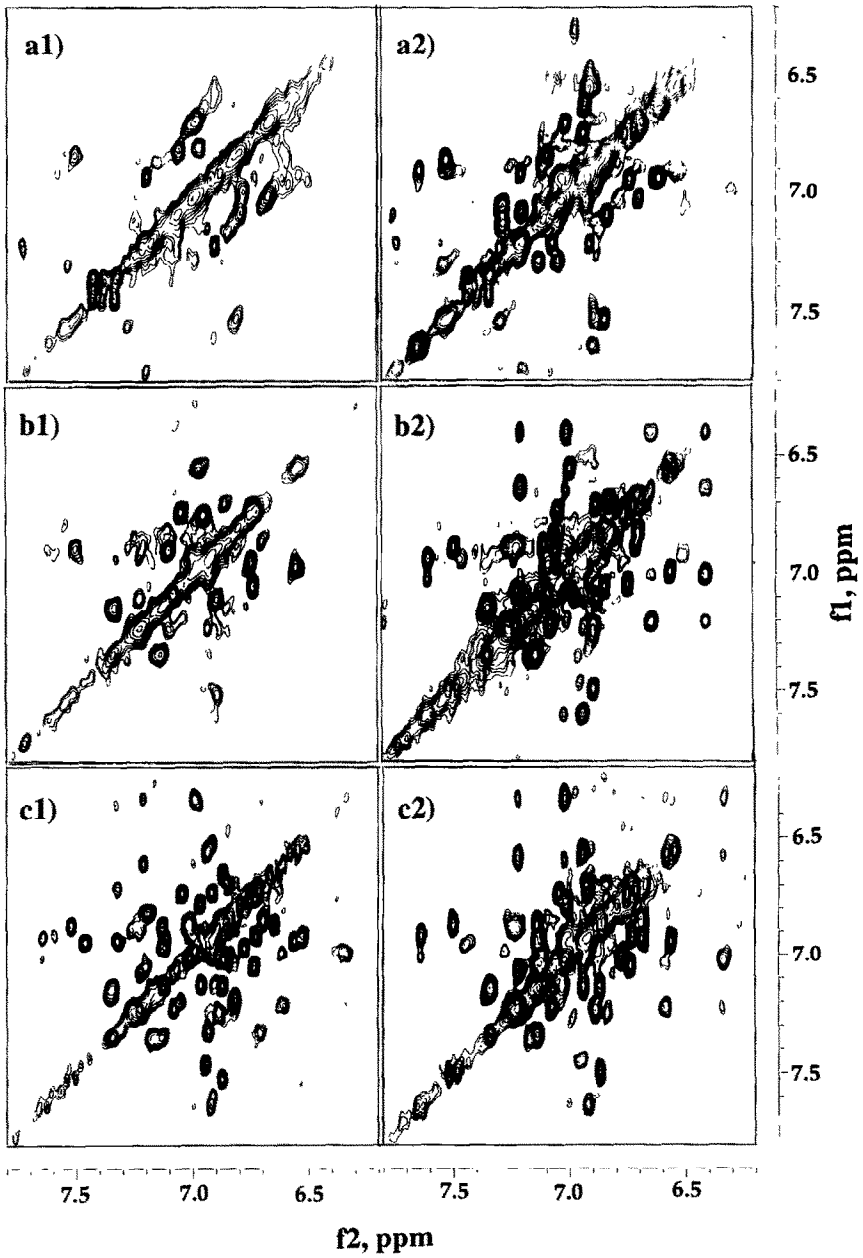


Fig. 2. Aromatic region of TOCSY 2D-spectra recorded in $D_2O$ with: (a1) Ox13-VH; (a2) Ox13-VH in the presence of 10 mM CHAPS; (b1) VH-P8; (b2) VH-P8 in the presence of 10 mM CHAPS; (c1) VH-P1; (c2) VH-P1 in the presence of 10 mM CHAPS.

Adding CHAPS, for example, did not improve the line width of the intact scFv αOx13 (J.D. and L.R., in preparation), in which the VH/VL interface was obviously not exposed to the solvent. In the case of the VH domains, CHAPS improved the $T_2$ of the two VH's with very broad lines. It had the greatest effect on the mutant, VH-P8, but no positive effect at all on VH-P1, which only differed from VH-P8 at a single position: residue 47 was an isoleucine in VH-P8 but a glycine in VH-P1. This indicated that residue VH-47 was critical for the nature of the exposed interface in the VH. This single point mutation also caused the movement of several chemical shifts in the aromatic region of VH-P8 and VH-P1 (Fig. 2).

For heteronuclear NMR experiments the mutant VH-P8 analysed in the presence of CHAPS would now be preferable to VH-P1 because of its better expression. In a 57 ms 2D-TOCSY experiment (Fig. 3) recorded with an $H_2O$ sample of VH-P8, magnetisation transfer from the amide to the side chain protons seemed sufficient for an assignment after labeling with $^{15}N$.

However, for other applications, like the design of non-sticky, minimum size immunoreagents, the VH-P1 mutant might be preferable as a starting point. Its long

relaxation time, which was not improved in the presence of CHAPS, suggested that there was little aggregation through the exposed VH/VL interface. In the case of the other two VH's this aggregation was strongly reduced through the addition of CHAPS. VH-P1 might, therefore, also be expected to cause less problems through non-specific binding in pharmaceutical or biotechnological applications.

### 3.4. Stability

Chemical shift movements in the aromatic region of the TOCSY spectra recorded with the wild-type Ox13-VH and VH-P8 (Fig. 2) were probably due to the mutation of residue VH-W47. However, numerous similarities to the aromatic region of the parent scFv (not shown) suggested that indeed all three VH's formed the normal immunoglobulin domain structure. This was further supported by their ability to bind protein A–Sepharose, which most likely depended on their proper tertiary structure. Thus protein A-conjugated peroxidase proved a very poor reagent for the detection of VH in Western blots of SDS gel denatured protein.

Other indicators for a correct tertiary structure were about 30 $D_2O$ stable amide protons observed for both the Ox13-VH and the VH-P8 mutant. Most of these exhibited TOCSY cross-peaks to downfield-shifted $C^{\alpha}H$'s (compare Fig. 3) indicative of a $\beta$-sheet arrangement [20], which was typical for an immunoglobulin domain and was observed for various Fv's analysed in our laboratory. No appreciable exchange was observed for these amides within several weeks.

In contrast amide protons of the VH-P1 mutant were almost completely exchanged already several hours after preparation of the $D_2O$ sample. VH-P1, however, exhibited the same downfield-shifted, $\beta$-sheet typical $C^{\alpha}H$ signals seen for the other two VH's. VH-P1 also formed a strong precipitate during the NMR experiments at 313 K. These preliminary observations hint at different stabilities of the VH's but require more detailed investigations. Results in this direction might become important for the design of stable VH domains as immunoreagents. Expression yields of VH's, equally important for biotechnological applications, could also be related to their stabilities.

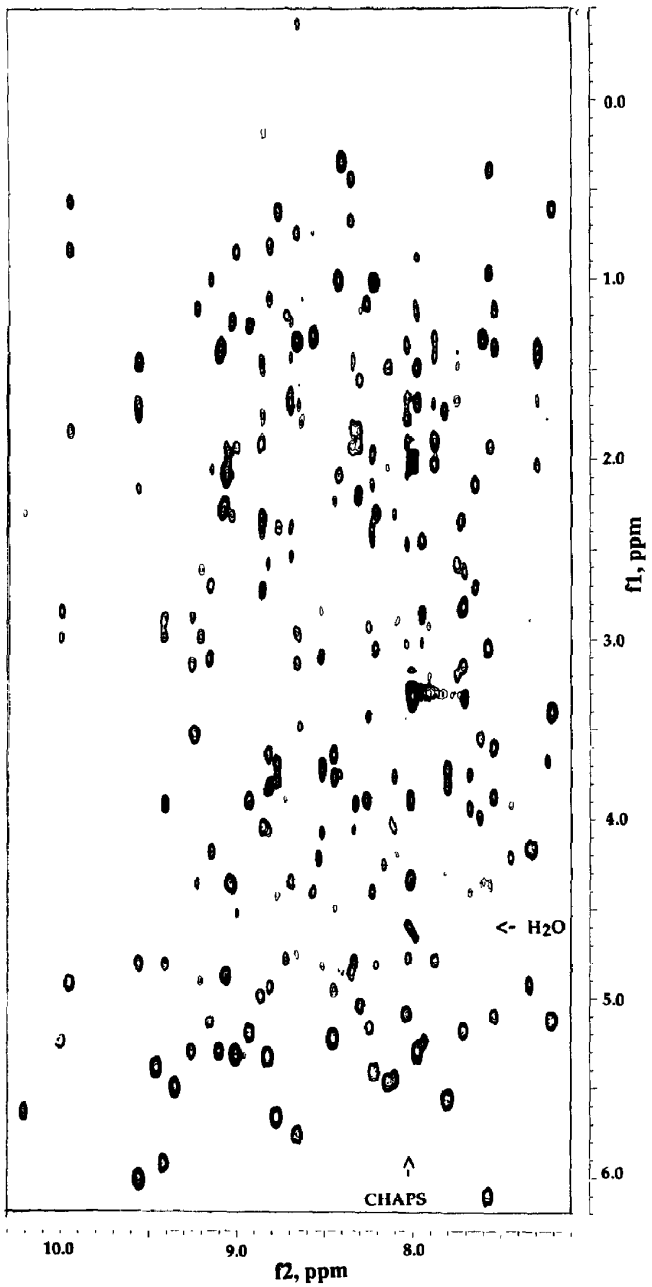


Fig. 3. Amide region of a TOCSY 2D-spectrum recorded in $H_2O$ with VH-P8 in the presence of 10 mM CHAPS.

## 4. Discussion

The engineering of VH domains as undertaken in this study should prove valuable in different aspects. The now possible NMR studies on an isotopically labeled VH-P8 domain should specifically ease the NMR analysis of the parent, scFv. More generally the achieved line width reduction showed how a protein, at first unsuitable for NMR analysis, can be studied when modified by protein engineering in structural regions likely to cause aggregation. This approach could be combined with an optimisation of sample conditions through the addition of detergent.

Similar actions might aid the NMR analysis of other proteins, where aggregation causes sensitivity problems through short relaxation times [17]. Proteins analysed by NMR spectroscopy are often expressed and analysed as fragments of originally much bigger macromolecules. Consequently surfaces or interfaces will be exposed to the solvent, which are hidden in the native protein. Added detergent or introduced mutations might therefore often render the studied macromolecule no less nat-

ural than before, although care will have to be taken with respect to effects on the overall structure.

Regarding the development of small immunoreagents, the use of VH's as minimum recognition units has been hampered by problems related to those observed during the NMR analysis. The exposed VH/VL interface can cause non-specific binding to hydrophobic ligands or surfaces. Irreversible denaturation at higher concentrations, as observed for the VH-P1 mutant, will be critical for scale up procedures in biotechnological applications. Similarly a synthetic, VH-like protein containing a metal-binding site proved insoluble at higher concentrations [21].

Changes in the VH/VL interface like the ones described here might solve these problems. For the design of small immunoreagents a library of VH's based on, for example, the non-aggregating VH-P1 mutant with randomised hypervariable loops (CDRs) can be expressed on the surface of phage and selected through binding to different immobilised antigens. Phage surface-expressed scFv libraries with randomised CDRs have already been successfully selected for different specificities [12] and improved affinity [22]. NMR analysis of resulting VH's would later be greatly simplified through comparisons with the VH-P8 domain, which has identical FR sequences, with the exception of residue VH-47.

Regarding the stability and expression yield of isolated VH's, both seemed to be highly sensitive to the mutations introduced in FR2. It will therefore be worthwhile analysing other mutants in our VH library (randomised at position 47) with respect to these parameters.

It should also be pointed out that the VH mutants were designed to match the sequences of camel VH's found in antigen binding immunoglobulins without light chains. The mutations analysed here might have enabled or even caused antibodies in camels to be expressed as immunoglobulins devoid of light chains, thus the FR2 mutations might prevent proper VH/VL interactions. This can be tested in association experiments of bacterially expressed heterodimeric Fv.

Antibodies devoid of light chains may also exist in species other than camel. Thus the Kabat database of immunoglobulin sequences [23] lists one frog VH family (VIII, *Xenopus laevis*), which exhibits mutations at positions VH-45 and VH-47 similar to those introduced in the human VH in our experiments. However, it has not been determined whether expressed members of this frog VH family form light chain-containing antibodies [24].

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