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Solution Properties of *Escherichia coli*-Expressed V_H Domain of Anti-Neuraminidase Antibody NC41

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The V_H domain of anti-influenza neuraminidase antibody NC41, with and without a C-terminal hydrophilic marker peptide (FLAG™), has been expressed in high yield (15–27 mg/L) in *Escherichia coli*. Both forms were secreted into the periplasm where they formed insoluble aggregates which were solubilized quantitatively with 2 M guanidine hydrochloride and purified to homogeneity by ion-exchange chromatography. The V_H-FLAG was composed of three isoforms (pI values of ~4.6, 4.9, and 5.3) and the V_H molecule was composed of two isoforms with pI values of 5.1 and 6.7; the difference between the V_H isoforms was shown to be due to cyclization of the N-terminal glutamine residue in the pI 5.1 isoform. At 20°C and concentrations of 5–10 mg/ml the V_H domain dimerized in solution and then partly precipitated, resulting in the broadening of resonances in its ^{1}H NMR spectrum. Reagents such as CHAPS, *n*-octylglucoside, and ethylene glycol, which presumably mask the exposed hydrophobic interface of the V_H molecule, prevented dimerization of the V_H and permitted good-quality NMR spectra on isotope-labeled protein to be obtained.

KEY WORDS: Antibody; V_H domain; dimerization; detergent stabilization of monomer; NMR analysis.

1. INTRODUCTION

Monoclonal antibodies, with their unique specificity and affinity, are widely used as immunodiagnostic and therapeutic reagents (Winter and Milstein, 1991). The cloning of antibody genes (Orlandi *et al.*, 1989) and their expression in mammalian (Neuberger *et al.*, 1984; Riechmann *et al.*, 1988) and bacterial cells (Skerra and Plückthun, 1988; Better *et al.*, 1988) has led to the production of a new range of recombinant antibody fragments. These recombinant antibodies offer considerable advantages over conventional monoclonal antibodies since they can be genetically manipulated to produce antigen-binding domains covalently attached to additional functional domains including enzymes, toxins, and epitopes of diagnostic interest (Haber, 1992). The Fv or single-chain fragment (scFv),[4] formed by joining the variable domains of

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[4] Abbreviations: CDR, complementarity-determining region; CHAPS, 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate; 2D-TOCSY, two-dimensional total correlation spectroscopy; FLAG, hydrophilic octapeptide tail, DYKDDDDK; Fv, antibody fragment containing variable domains; GuHCL, guanidine hydrochloride; HRP, horseradish peroxidase; M_r, relative molecular mass; PBS, phosphate-buffered saline, *p*H 7.3; scFv, single-chain Fv fragment; V_H and V_L, variable domains of antibody heavy and light chains, respectively.

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heavy chain (V_H) and light chain (V_L) with a peptide linker (Huston *et al.*, 1991), is the smallest fragment produced which shows binding affinities comparable to the parent Fab and has been used for a number of clinical and diagnostic applications (Lilley *et al.*, 1994). Attempts to produce single-domain antigen-binding fragments with affinities approaching the parent antibody have had limited success (Plückthun, 1990; Ward *et al.*, 1989; Power *et al.*, 1992). V_H domains with binding affinities of $\sim 10^6$ M for lysozyme have been obtained from expression libraries of mouse V_H genes (Ward *et al.*, 1989). However, the expression of isolated V_H or V_L domains has not produced molecules with the binding specificity or affinity comparable to the parent antibody. It is therefore important to correlate this observation with the structural properties of the V_H and V_L domains in isolation.

Individual recombinant antibody domains such as V_H domains with $M_r \sim 13{,}000$ are suitable for structural analysis by NMR methods using a variety of multidimensional and multinuclear approaches. Aggregation problems, especially at higher protein concentrations, however, have hampered NMR studies of these molecules. A human V_H domain, OX13 V_H, produced in Escherichia *coli* was found to aggregate, probably via the exposed hydrophobic surface on the V_H domain that interacts with the V_L domain (Davies and Riechmann, 1994). This aggregation problem was overcome by mutation of specific hydrophobic interface residues to hydrophilic residues that are found in this region in the heavy-chain gene sequence of camelid V_H domains. Camelid heavy chains occur in solution without light chains, which may account for the higher solubility of 'camelized' V_H domains (Hammers-Casterman *et al.*, 1993). A soluble V_L domain has been isolated and its solution structure determined by NMR analysis (Constantine *et al.*, 1994).

The V_H domain of the monoclonal antibody NC41, which recognizes an epitope on influenza virus neuraminidase, has been expressed in *E. coli* using a temperature-inducible expression system (Power *et al.*, 1992). V_H domains with and without a hydrophilic octapeptide affinity label, referred to as FLAG, fused at the C-terminus have been purified at yields of 15–27 mg/L. The three-dimensional structure of the complex between N9 neuraminidase and the NC41 Fab fragment has been determined by X-ray crystallography (Colman *et al.*, 1987, 1989; Tulip *et al.*, 1992) and the V_H domain of NC41 represents an opportunity for a comparative study by NMR methods of the structure, dynamics, and function of an isolated immunoglobulin domain in solution. In this paper we report on the purification, properties, and aggregation state of the NC41 V_H domain with and without the C-terminal FLAG peptide and on the preliminary results of NMR analysis of this V_H domain.

2. MATERIALS AND METHODS

2.1. Expression and Isolation of the NC41 V_H Domain

NC41 V_H gene constructs with and without a C-terminal FLAG affinity label were expressed using a temperature-inducible vector (pPOW) as described previously (Power *et al.*, 1992). Expression in *E. coli* strain BMH 71-18 yielded the bulk of the V_H product, from both constructs, in the periplasm as an insoluble aggregate which associated with the cell membrane pellet on lysis of the cells by sonication; a small amount of soluble V_H (and V_H-FLAG) was found in the supernatant fraction. These membrane and supernatant fractions were prepared as described previously (Power *et al.*, 1992).

2.2. Purification of V_H

The membrane pellet from 1 L of culture was resuspended in 60 ml of 2.0 M guanidine hydrochloride (GuHCl), 0.1 M Tris-HCl, *p*H 8.0, and stirred for 2 hr at room temperature. The suspension was centrifuged to remove insoluble residue and the supernatant containing the solubilized V_H was dialyzed extensively against 0.025 M Tris-HCl, *p*H 8.0, at 4°C to remove GuHCl. A precipitate that formed on dialysis was removed by centrifugation and the soluble fraction was concentrated by ultrafiltration (Amicon stirred cell, YM10 membrane), and chromatographed on a Mono-Q (HR5/5) column in 0.025 M Tris-HCl, *p*H 8.0, using a linear salt gradient to elute symmetrical peaks of V_H-FLAG and V_H.

Soluble V_H and V_H-FLAG present in the supernatant fraction were purified as follows: the supernatant was concentrated ~10-fold by ultra-

filtration and passed down a Sephadex G-100 column (50 × 2 cm) in 0.025 M Tris-HCl, 0.1 M NaCl, *p*H 8.0, to remove high- and low-molecular-mass bacterial proteins. Fractions containing V_H were pooled, concentrated, and chromatographed on a Mono-Q column as described above.

2.3. Large-Scale Purification of V_H

For large-scale purification of V_H (without FLAG), *E. coli* BMH 71-18 cells were grown in a 2-L fermenter (LH series 500, LH Engineering, U.K.), using a defined medium with ammonium chloride as the nitrogen source and glycerol as the carbon source. $^{15}NH_4Cl$ (99.7 at % ^{15}N) was purchased from Isotec Inc. (Ohio). The temperature was maintained at 28°C prior to induction and the *p*H was controlled at 7.0 using 4 N NaOH and 2 N H_2SO_4. Foam was controlled with LQ217 anti-foam (Hemkel, Australia). Induction was carried out at an A_{600} of 4.0 by raising the temperature of the culture to 42°C as described previously (Power *et al.*, 1992). The culture was harvested 4 hr after induction at an A_{600} of 6.0. The cells were centrifuged at 5000*g* and the pellet was resuspended in PBS at *p*H 7.3. The cells were lysed using sonication (Branson Sonic Power Co.) and centrifuged at 12,000*g* for 45 min to yield the soluble and membrane fractions.

The cell membrane pellet from 2 L of culture was extracted with 90 ml of 2.0 M GuHCl, 0.1 M Tris-HCl, *p*H 8.0, the residue was reextracted with 90 ml of the same buffer, and the supernatants dialyzed at 4°C against 6 × 2 L of deionized water. The dialyzates were warmed to room temperature and centrifuged at 7740*g*, 20°C for 15 min. The supernatant was adjusted to *p*H 8.8 with Tris buffer and applied to a Merck Fractogel EMD TMAE-650 column (86 × 16 mm) equilibrated with 0.025 M Tris-HCl, *p*H 8.8, at room temperature. The column was eluted with the starting buffer at 3 ml/min for 10 min and then a linear gradient of NaCl to 0.07 M NaCl in 20 min was applied. Fractions containing V_H were dialyzed against water, concentrated by ultrafiltration (Amicon YM10 membrane), and stored frozen. Samples for NMR analysis were chromatographed on a Sephacryl S-100 column (2.6 × 90 cm) in PBS at a flow rate of 2.5 ml/min and the fractions containing V_H were concentrated to ~10 mg/ml. The ^{15}N-labeled V_H was purified using the same protocol. The yield of purified V_H was ~27 mg/L using fortified superbroth medium described in Power *et al.* (1992) and ~15 mg/L using the defined medium as described above.

2.4. Other Chromatographic Methods

The two isoforms of V_H were separated by chromatography on a Mono-S (HR5/5) column equilibrated with 0.025 M sodium acetate, 0.09 M NaCl, *p*H 4.2, and eluted with a linear gradient to 0.14 M NaCl at a flow rate of 1 ml/min. The two V_H peaks were dialyzed against water and lyophilized.

The molecular mass and purity of the V_H preparations were determined by analytical gel filtration on a calibrated Superose 12 column (30 × 1 cm) in phosphate-buffered saline (PBS) run at a flow rate of 0.5 ml/min. The column was calibrated with the following standard proteins: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin dimer (132 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), and soybean trypsin inhibitor (21.5 kDa).

2.5. Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed in 1.5-mm slabs [15% (w/v) polyacrylamide/0.8% bis] in a Bio-Rad Mini-Protean II gel apparatus (Laemmli, 1970). Polyacrylamide gel electrophoresis under nondenaturing conditions was carried out in 1.5-mm slabs [7.5% (w/v) polyacrylamide] at *p*H 8.8 (Davis, 1964; Ornstein, 1964). The gels were stained with 0.2% Coomassie brilliant blue G in 10% (v/v) acetic acid/50% methanol and destained in 7.5% (v/v) acetic acid/10% methanol. Isoelectric focusing was carried out using Ampholine (LKB) polyacrylamide gel plates (*p*H range 3.5–9.5) according to the manufacturer's instructions, using an LKB Multiphor apparatus at 10°C for 5 hr with a maximum current of 10 mA and a final voltage of 1400 V. The plates were stained with Coomassie blue R (0.1 g/L) containing $CuSO_4$ (1 g/L) in 10% acetic acid/25% ethanol (Righetti and Drysdale, 1974). For Western blots, proteins were transferred to nitrocellulose with 0.025 M Tris-glycine, *p*H 8.5, containing 20% methanol as the transfer buffer. The detection reagent was sheep anti-mouse HRP conjugate (Silenus) as the second antibody and 4-chloro-1-naphthol as the color reagent.

2.6. Tryptic Cleavage and Peptide Fractionation

V_H and V_H-FLAG samples (~150 μg) were dissolved in 200 μl of 0.1 M ammonium bicarbonate and digested with trypsin (5 μg) at 37°C for 3 hr. The digests were dried at 50°C under vacuum and redissolved in 0.5 ml of 0.1% trifluoroacetic acid and centrifuged. The peptides were fractionated by RP-HPLC on a Vydac 218 TP54 column (4.4 × 250 mm) as described previously (Caldwell *et al.*, 1990).

Tryptic peptide, T1a, which had a blocked N-terminus, was digested with pyroglutamyl aminopeptidase (Boehringer-Mannheim) as described (Crimmins *et al.*, 1988). The digest was purified by RP-HPLC using the same conditions as for the original digest, to yield a main peak eluting at a lower concentration of acetonitrile than the undigested peptide.

Peptide sequences were determined with a Model 470A gas-phase sequencer (Applied Biosystems, U.S.) with on-line phenylthiohydantoin (PTH) analysis. Proteins and peptides (~3 nmol) for amino acid analysis were hydrolyzed in 6 M HCl (24 hr) at 110°C and analyzed on a Waters HPLC amino acid analyzer using postcolumn ninhydrin detection.

2.7. V_H Aggregation

The state of aggregation of V_H and V_H-FLAG stored under various conditions was monitored by FPLC on Superose 12 in 0.025 M sodium acetate, 0.05 M NaCl, *p*H 5 (flow rate 0.5 ml/min), and by SDS–PAGE with and without β-mercaptoethanol. Aggregated V_H samples were fractionated into monomeric and dimeric forms by chromatography on Superose 12 and concentrated to 5.4 and 2.3 mg/ml, respectively. The stability of the monomer and dimer V_H was assessed over 14 days in the presence of a number of detergents and reagents that affect aggregation, including 3-[(cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) (17 mM), *n*-octylglucoside (17 mM), ethylene glycol (60% v/v), urea (1.7 M), and KCl (1 M). The V_H samples in the presence of each reagent were stored at ambient temperature (30 ± 2°C) and 5-μl aliquots were taken for Superose 12 chromatography to monitor the stability of the monomer and dimer forms. The heights of the peaks in the chromatograms were measured and used to calculate the percentage of dimer to monomer in each run. In a second experiment the effect of ethylene glycol (5%, 10%, and 30% v/v) was examined at ambient temperature with a V_H sample at 8.2 mg/ml. The effect of storage of V_H at 4°C was also examined. Similar experiments were also performed with V_H-FLAG.

2.8. Circular Dichroism Measurements

Circular dichroism (CD) experiments were performed with an Aviv 62 DS Circular Dichroism Spectrometer at 20°C, using V_H concentrations of ~1 mg/ml in PBS and cells of 1 mm path length.

2.9. Binding Studies

Studies to measure the interaction of NC41 V_H with tern and whale N9 neuraminidase included sedimentation equilibrium analysis using a Beckman airfuge with ^{125}I-labeled V_H-FLAG as described (Gruen *et al.*, 1993) and biosensor analysis using the IAsys biosensor (Fisons Applied Sensor Technology) with neuraminidase immobilized on the cuvette surface. The binding activity of NC41 V_H toward tern N9 neuraminidase was also assessed qualitatively by size exclusion FPLC.

2.10. NMR Spectroscopy

NMR studies were carried out with a protein concentration of ~1 mM (15 mg/ml) in 90% H_2O/10% ^2H_2O in the presence or absence of 17 mM CHAPS, at *p*H 5.5 with a total sample volume of 500 μl. The ^{1}H NMR spectra were recorded at 20°C on a Bruker AMX-600 spectrometer. The carrier was placed in the center of the spectrum and quadrature detection was used. Homonuclear 2D-NMR spectra were recorded in phase-sensitive mode using time-proportional phase incrementation (Marion and Wüthrich, 1983). TOCSY spectra (Braunschweiler and Ernst, 1983; Bax and Davis, 1985) were recorded using a DIPSI-2 (Rucker and Shaka, 1989) spin lock mixing sequence and a mixing time of 30–50 msec, at a spin lock field strength of 9.8 kHz. Heteronuclear ^{1}H–^{15}N correlated spectra were acquired using the standard pulse sequences of Bax *et al.* (1983) and Bodenhausen and Ruben (1980). Decoupling of ^{15}N during acquisition was performed with the composite pulse sequence GARP-1 (Shaka *et al.*, 1985) with a field strength of 1.6 kHz.

Solvent suppression was achieved using phase-coherent presaturation during the relaxation delay (2 sec). The ^1H spectral width was 7042 Hz, acquired over 2K data points and 256–512 increments with 128 scans per increment. The ^{15}N spectral width was 2500 Hz. Data were zero-filled to yield final 2K × 2K real matrices. Before Fourier transformation a phase-shifted, sine-bell squared apodization was applied, shifted 60° in ω_1 and 45° in ω_2. Data processing and analysis were carried out using the software packages UXNMR and AURELIA (Bruker Analytische Messtechnik GmbH).

3. RESULTS AND DISCUSSION

3.1. Purification

The pPOW expression system in *E. coli* strain BMH 71-18 yielded V_H and V_H-FLAG mainly as insoluble protein aggregates in the periplasm and the protein pelleted with the cell membrane fraction on centrifugation of disrupted cells. About 90% of the synthesized protein was solubilized by a single extraction with 2 M GuHCl, 0.1 M Tris-HCl, *p*H 8.0 buffer, and V_H and V_H-FLAG remained in solution on removal of the denaturant by dialysis. The hydrophilic C-terminal FLAG peptide did not enhance the solubility of the expressed V_H. Chromatography of the solubilized V_H (and V_H-FLAG) on a Mono-Q column yielded a peak of pure protein.

Preparative-scale chromatography of GuHCl-solubilized V_H extracts on Fractogel EMD TMAE-650 gave essentially pure V_H (Fig. 1). The Sephacryl S-100 column removed V_H aggregates and low-molecular-mass contaminants that interfered with the NMR spectra.

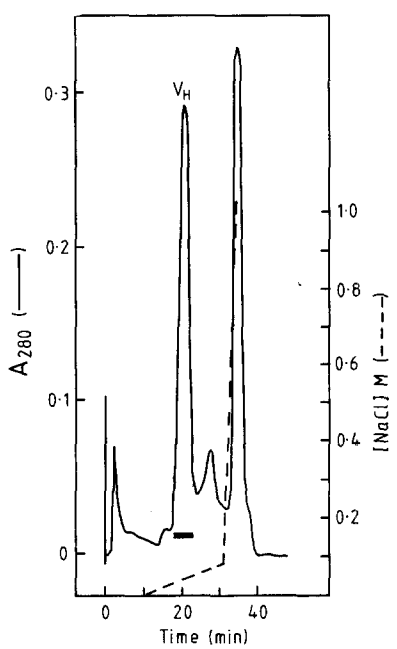


Fig. 1. Chromatography of solubilized V_H on a column (86 × 16 cm) of Fractogel EMD TMAE-650M (Merck). The sample (130 ml of soluble extract containing ~20 mg of protein) was loaded onto the column and eluted with 0.025 M Tris-HCl, *p*H 8.8, for 10 min and then a linear gradient to 7% of 0.025 M Tris-HCl, 1.0 M NaCl, *p*H 8.8, over 30 min at a flow rate of 3 ml/min was applied. The V_H peak indicated by the bar was collected.

3.2. Purity and Isoelectric Point

The V_H and V_H-FLAG preparations were homogeneous as judged by nondenaturing PAGE at *p*H 8.8 (Fig. 2A) and SDS–PAGE (Fig. 2B). N-terminal analyses gave the expected sequence, and the amino acid compositions of both V_H and V_H-FLAG were in close agreement with the calculated compositions. Gel filtration on a calibrated Superose 12 column showed that the

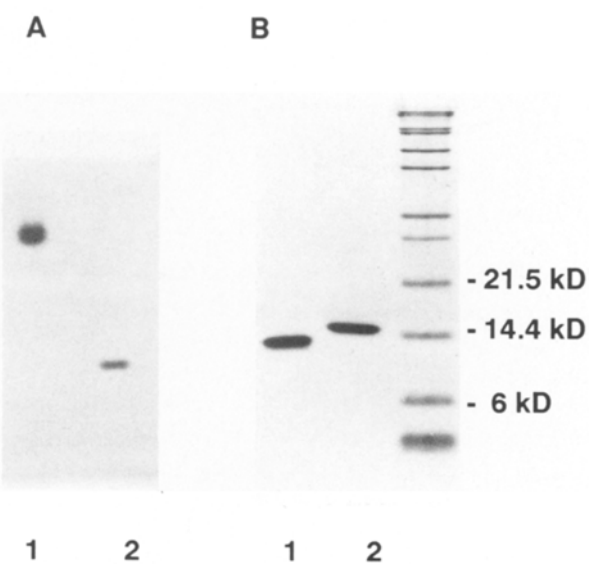


Fig. 2. Polyacrylamide gel analysis of purified V_H. (A) native PAGE at *p*H 8.8 (7.5% polyacrylamide): lane 1, V_H; lane 2, V_H-FLAG. (B) SDS–PAGE (15% polyacrylamide): lanes 1 and 2 as for panel A.

purified V_H preparations eluted as monomers with a molecular mass of ~14 kDa.

Isoelectric focusing, however, showed that both purified V_H and V_H-FLAG were not single components (Fig. 3). V_H-FLAG focused as three bands with pI values of ~4.6, 4.9, and 5.3, whereas V_H focused as two bands with pI values of 5.1 and 6.7 (Fig. 3). The two components present in the V_H preparation were separated by cation exchange chromatography on a Mono-S column (Fig. 4). The amino acid compositions of these two components were identical and hence they are isoforms of V_H. The pI 6.7 isoform gave the expected N-terminal sequence, QVQLQQSGPELK, but the pI 5.1 isoform was blocked. As the N-terminus of the V_H is glutamine, cyclization to pyroglutamic acid would account for the blocked N-terminus and the lower pI due to the loss of a positive charge.

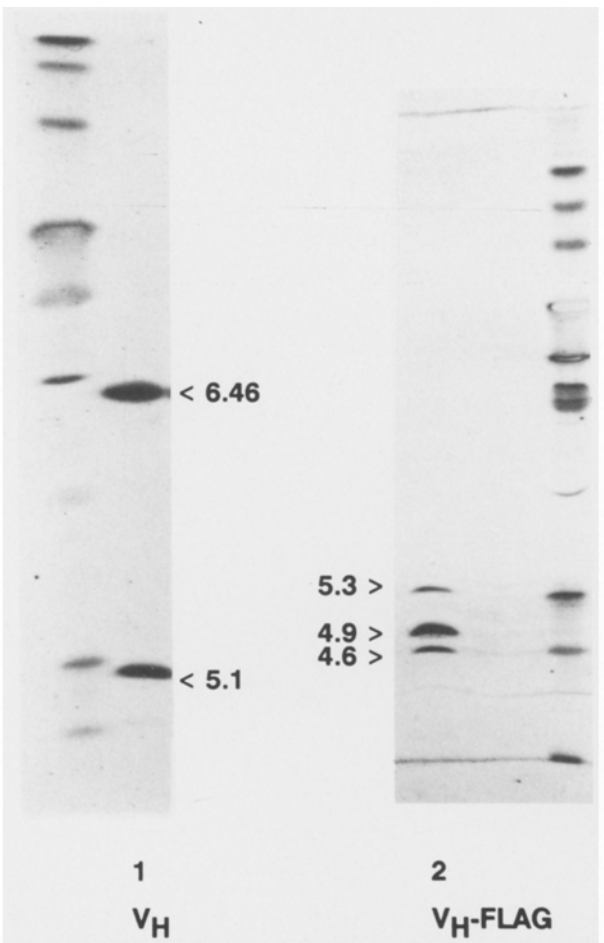


Fig. 3. Isoelectric focusing of V_H and V_H-FLAG. Left panel shows the V_H and the right panel shows the V_H-FLAG in separate focusing experiments.

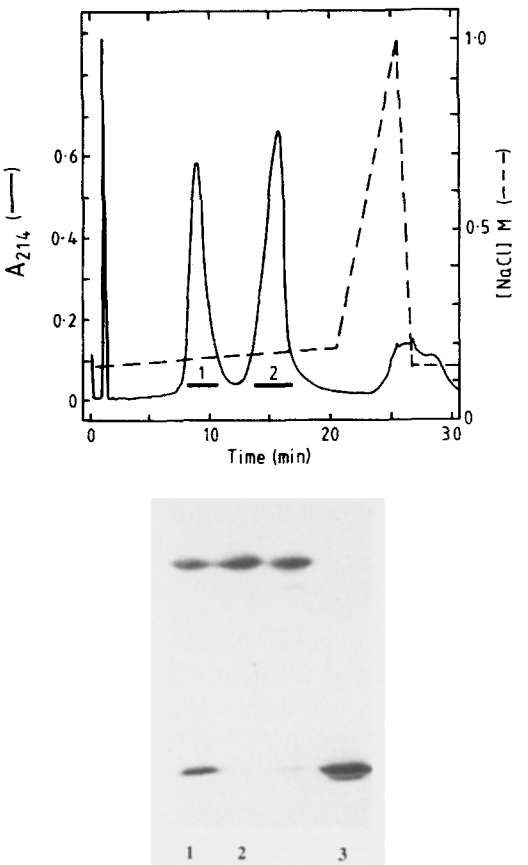
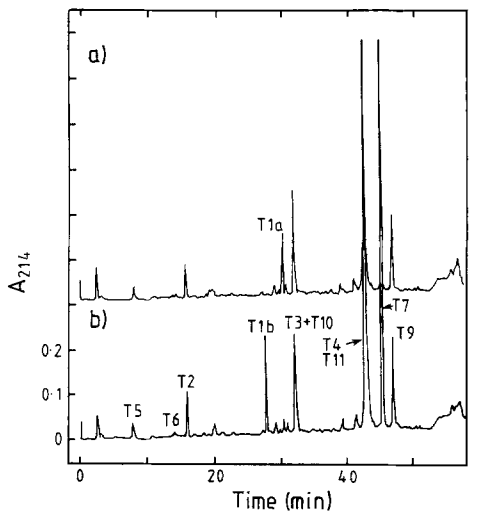


Fig. 4. Chromatography of V_H on Mono-S HR5/5 column (Pharmacia-LKB) to separate the two V_H isoforms. The column was equilibrated with 0.025 M sodium acetate, 0.09 M NaCl, *p*H 4.2, and eluted with a linear gradient to 0.14 M NaCl at a flow rate of 1 ml/min. The two peaks were pooled as indicated by the bars. The bottom panel shows the isoelectric focusing gel of V_H and the two separated isoforms. (1) V_H, (2) pI 6.7 isoform. (3) pI 5.1 isoform.

Comparison of peptide profiles of tryptic digests of the two V_H isoforms showed only one difference in the peptide positions (Fig. 5a). The tryptic peptide peaks in Fig. 5a were identified by sequencing and amino acid analysis as indicated in the sequence in Fig. 5b. Peptides T1a and T1b (Fig. 5a) gave the amino acid composition expected for the N-terminal tryptic peptide of V_H and the peptide (T1a) from isoform pI 5.1 was blocked. Pyroglutamyl aminopeptidase digestion of blocked peptide T1a gave a single peptide which on RP-HPLC eluted from the column earlier than T1a and yielded the N-terminal sequence VQL, indicating that it was the deblocked N-terminal peptide. These results show that cyclization of the N-terminal glutamine residue accounts for the



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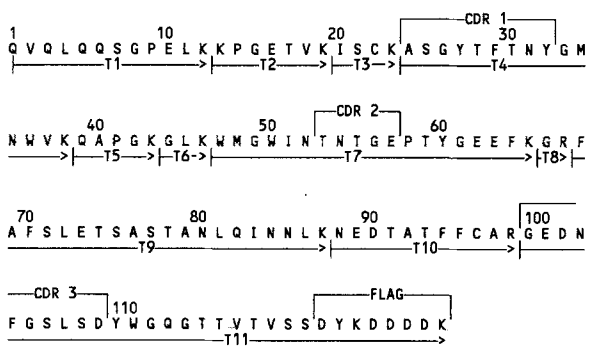

Fig. 5. Reverse-phase HPLC profiles of tryptic digests of (a) V_H isoform pI 5.1 and (b) V_H isoform pI 6.7. The Vydac 218 TP54 column (4.4 × 250 mm) was equilibrated in 0.1% (by volume) trifluoroacetic acid at 45°C and peptides eluted with a linear gradient of 0.1% (by volume) trifluoroacetic acid/70% acetonitrile over 60 min. The tryptic peptides were identified by sequencing and amino acid analysis as indicated below. (c) The amino acid sequence of recombinant V_H-FLAG domain showing the tryptic peptides identified in Fig. 5a, b by amino acid composition and sequencing.

isoforms observed for the purified V_H, although it is not known at which stage of the expression or purification this cyclization occurs. The tryptic peptide profile of V_H-FLAG contained both peptides T1a and T1b, indicating that some of the V_H-FLAG molecules also have a cyclized N-terminal glutamine. This accounts for two of the bands observed on isoelectric focusing, but the origin of the third band was not investigated (Fig. 3). The profile for V_H-FLAG was identical to the tryptic peptide profile for V_H except that T11, which now contains the FLAG peptide, separates from T4. Trypsin did not cleave the Lys–Asp bond in the C-terminal FLAG peptide.

3.3. V_H Aggregation

At alkaline *p*H (~11.0) V_H dimerized slowly and higher-molecular-mass multimers were formed on extended storage. SDS–PAGE under nonreducing and reducing conditions showed (data not shown) that this aggregation was due to disulfide cross-linking, presumably due to disulfide interchange. This covalent dimerization was not observed at acid *p*H.

In PBS at 1 mg/ml, V_H and V_H-FLAG molecules were stable at 4°C and room temperature and showed no signs of aggregation. However, at protein concentrations of 5–10 mg/ml, as required for NMR measurements, the V_H started to form dimers in solution and eventually to precipitate (see below). Superose 12 gel filtration of V_H samples after NMR analysis showed that considerable dimerization had occurred (Fig. 6).

The effect of temperature, detergents, and denaturants on the dimerization of V_H at higher protein concentrations was therefore investigated. The V_H monomer at 5.4 mg/ml and 4°C did not dimerize, whereas at 20°C the monomer slowly dimerized to yield about 30% dimer after 14 days (Fig. 7a). In contrast, the V_H dimer was relatively stable, with only about 5% dissociating over this time period. Low concentrations of urea (1.7 M) accelerated both dimerization of the monomer and dissociation of the dimer to an equilibrium mixture

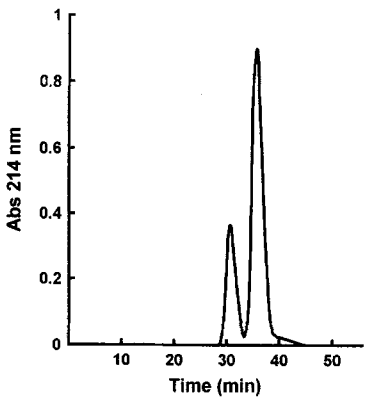

Fig. 6. Superose 12 gel filtration of a V_H monomer sample at 10 mg/ml after NMR analysis. The column was equilibrated with 0.025 M Na acetate, 0.05 M NaCl, *p*H 5.0, and run at a flow rate of 0.5 ml/min. The peak eluting at 30.3 min is dimer and that at 35.2 min is monomer.

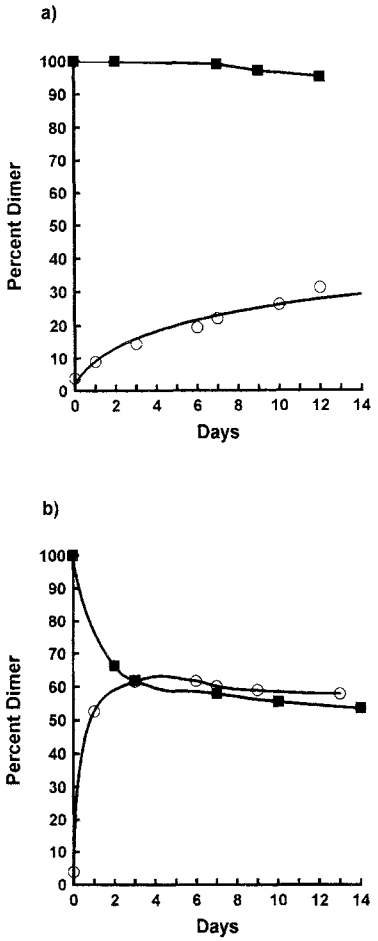

Fig. 7. Stability of V_H monomer and dimer. (a) Monomer (5.3 mg/ml) (O-O) and dimer (2.8 mg/ml) (■-■) were stored at room temperature (~20°C) and the proportions of monomer to dimer in the sample determined by gel filtration on Superose 12. (b) Effect of urea (1.7 M) on the stability of V_H monomer (O-O) and dimer (■-■) under the same conditions.

of about 40% monomer and 60% dimer in 3 days (Fig. 7b). KCl (1 M) also accelerated dimer formation (~50% formed in 14 days), but had little effect on dissociation of the dimer. Reagents which disrupt hydrophobic interactions, such as CHAPS (17 mM), *n*-octylglucoside (17 mM), and ethylene glycol (≥10% v/v), at 20°C stabilized the monomer, with no dimerization evident after 14 days (Fig. 8). *N*-Octylglucoside did not dissociate the dimer, whereas CHAPS and ethylene glycol produced partial dissociation (not shown). These results show that CHAPS and ethylene glycol at relatively low concentrations effectively prevent dimerization of the V_H domain at higher protein concentrations. The hydrophilic peptide (FLAG) at the C-terminus of the V_H domain did not affect the dimerization

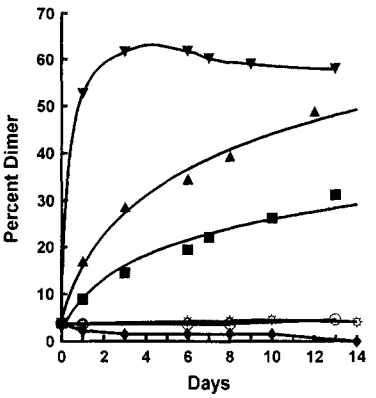

Fig. 8. Effect of detergents and other reagents on the dimerization of V_H monomer at 5.3 mg/ml and room temperature. (■) V_H, no additives; (▲) KCl (1 M); (▼) urea (1.7 M); (◆) ethylene glycol; (☼) CHAPS (1.7 M); (○) *n*-octylglucoside (1.7 M).

properties of the V_H-FLAG, as this molecule showed similar behavior to the V_H under the same conditions as described above.

Attempts to decrease the hydrophobicity of the exposed V_H surface by substituting Glu for Trp at residue 110 (Fig. 5c; residue 103 in Kabat numbering) failed to produce a molecule with improved solubility characteristics. This mutant (V_H-W110E) expressed to similar levels as the V_H, but on extraction was found to aggregate extensively (Power *et al.*, unpublished data).

3.4. Circular Dichroism Spectrum

The far-ultraviolet CD spectrum of an aqueous PBS solution of soluble V_H isolated from the *E. coli* supernatant is shown in Fig. 9. The spectrum features a broad negative band with λ_{max} about 217 nm, which is characteristic of β-sheet-rich proteins (Brahms and Brahms, 1980). The absence of strong negative bands centered around 208 and

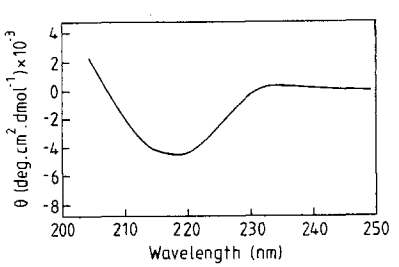

Fig. 9. Circular dichroic spectrum of NC41 V_H domain.

222 nm indicates that the α-helix content of the antibody fragment is minimal. The secondary structure of V_H was predicted from this CD spectrum using the estimator program PROSEC, which uses basis spectra derived from Yang *et al.* (1986); this analysis revealed that ~75% of the V_H molecule exists in the β-conformation, with only ~6% present as α-helix. CD spectra of the soluble V_H-FLAG and that of V_H-FLAG extracted with GuHCl were virtually identical, thus indicating that any partial unfolding in 2 M GuHCl and subsequent refolding of the molecule produced no apparent effect on the secondary structure.

3.5. Binding Studies

Gel filtration on a calibrated Superose 12 column in PBS showed no detectable interaction between V_H-FLAG and N9 tern neuraminidase. Sedimentation equilibrium experiments with ^{125}I-labeled V_H-FLAG also indicated that binding to neuraminidase was extremely weak and a K_a of $<10^4\ M^{-1}$ was estimated. No V_H-FLAG binding to immobilized tern N9 neuraminidase was detected using an IAsys biosensor instrument; in contrast, NC41 V_L was found to bind weakly to immobilized neuraminidase in the biosensor (D. Dougan, unpublished results).

The NC41 V_H domain was not recognized by anti-idiotype antibody 1C9-10, which binds strongly to the parent NC41 Fab. These results indicate that in the case of this antibody, the three CDR regions of the V_H domain are not sufficient for detectable binding to the epitope on neuraminidase or recognition by the anti-idiotype antibody and require a combined contribution from the CDRs of the V_L domain. Recombinant anti-neuraminidase scFv's of antibodies NC41 and NC10 bind strongly to neuraminidase and anti-idiotype antibodies against the parent antibodies (Kortt *et al.*, 1994; Kortt *et al.*, unpublished data). Structural studies (Tulip *et al.*, 1992) have shown that in the interaction between tern N9 neuraminidase and NC41 Fab only five of the six CDRs make contact with the neuraminidase over a large surface area, with the V_H domain contributing three CDRs and the V_L only two CDRs. Therefore, contribution of the V_L CDRs is essential for binding to occur with this antibody molecule. It is possible, however, that the structure of the V_H domain, particularly in the vicinity of the combining site, may be altered in the recombinant molecule.

3.6. NMR Analysis of the NC41 VH Domain

The 600-MHz 1D ^{1}H-NMR spectrum of the V_H domain in H_2O at 20°C and *p*H 5.5 is shown in Fig. 10 and a homonuclear 2D TOCSY spectrum in Fig. 11. The heteronuclear 2D ^{1}H–^{15}N correlated NMR spectrum of uniformly ^{15}N-labeled V_H domain shown in Fig. 12 displays well-dispersed nitrogen and amide proton chemical shifts. The number of signals displayed in the ^{1}H–^{15}N correlated spectrum and the number of correlations in the fingerprint region of the TOCSY spectrum (Fig. 11) are appropriate for a protein of the size of the V_H domain. The chemical shift dispersion of both the amide protons and nitrogens in the ^{1}H–^{15}N correlated spectrum, in conjunction with a large dispersion of chemical shifts for resonances of the α-protons in the homonuclear spectra, is consistent with the presence of β-sheet secondary structure. Slowly exchanging amide protons in an ^2H_2O exchange experiment are also indicative of the presence of secondary structural elements; 22 such amide protons remain unexchanged 11 days after dissolving the V_H protein in ^2H_2O. The features demonstrated by the NMR spectra indicate that the isolated V_H domain exists in solution as a stable folded entity rather than in a random coil conformation and the spectral features are consistent with the presence of β-sheet secondary structure as would be expected for this antibody domain.

In the course of the NMR experiments a protein precipitate appeared, accompanied by a significant degradation of spectral quality. This deterioration in the NMR spectra of the V_H domain manifested as an increase in resonance linewidths and a reduction of signal intensity, rendering these spectra difficult to interpret. PAGE analysis of the NMR samples demonstrated that no significant proteolytic degradation of the protein had occurred, but gel filtration showed the formation of dimer in the sample (Fig. 6) over the time period of the experiment. Protein aggregation causes an increase in the rotational correlation time of the protein, which is reflected in NMR spectra by an increase in the inherent linewidths of resonances (Wüthrich, 1986). Dimerization of another V_H

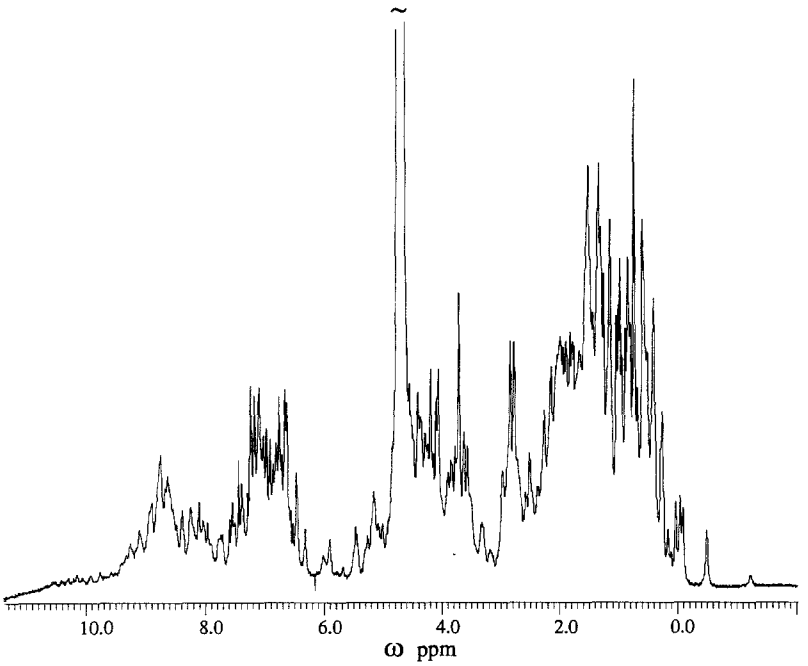

Fig. 10. The 1D 600-MHz ^{1}H-NMR spectrum of V_H domain in 90% H_2O/10% ^2H_2O at *p*H 5.5 and 20°C.

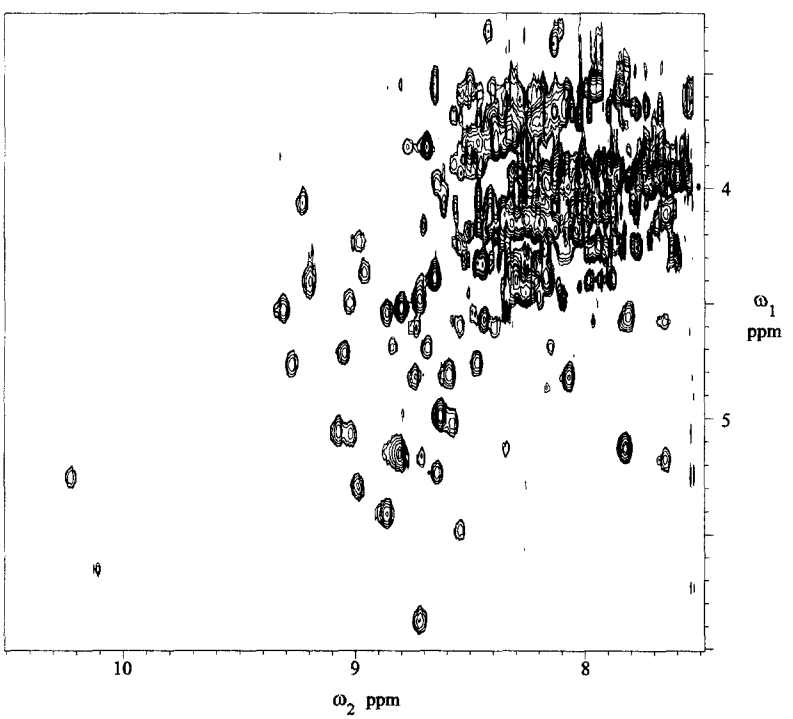

Fig. 11. The 2D TOCSY spectrum of the amide-alpha region of V_H domain at 44 msec mixing time acquired at *p*H 5.5 and 20°C in 90% H_2O/10% ^2H_2O. Downfield signals visible in the HMQC (Fig. 12) are visible at lower contour level.

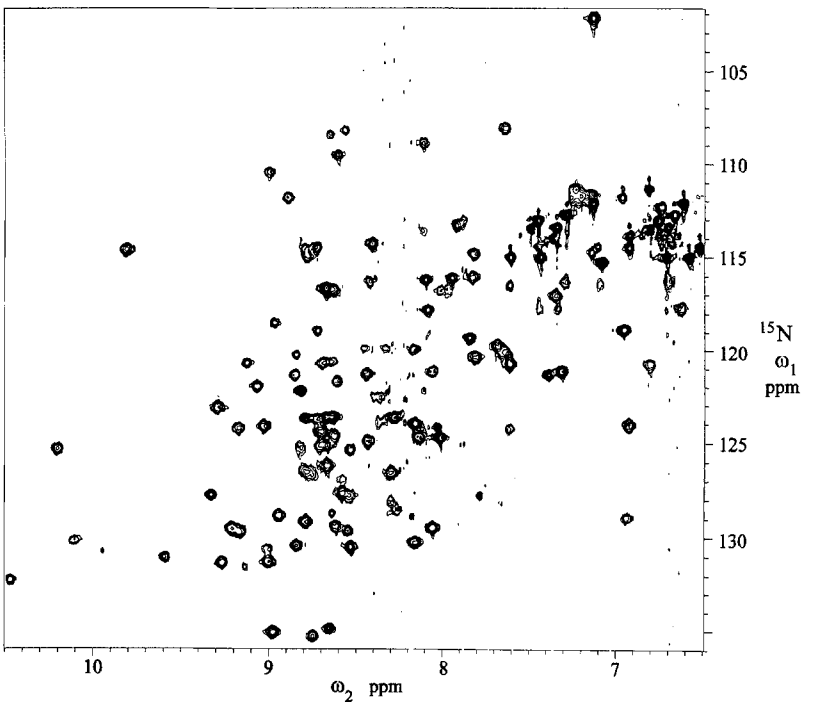

Fig. 12. The 2D ^1H–^{15}N correlation spectrum (HMQC) of V_H domain at 600 MHz in 90% H_2O/10% ^2H_2O at *p*H 5.5 and 20°C.

under similar conditions has been noted recently (Davies and Riechmann, 1994).

The problem of poor spectral quality was overcome by using conditions which prevent dimerization of the V_H domain. Careful temperature control and the use of detergents were shown to increase the stability of the monomeric V_H species in solution (Fig. 9). The detergent CHAPS has been used successfully to prevent sample aggregation and to improve NMR spectra by reducing the linewidth of resonances in other proteins (Anglister *et al.*, 1993; Davies and Riechmann, 1994). It is a mild nondenaturing detergent (Hjelmeland *et al.*, 1983) which is thought to alleviate aggregation by binding to exposed hydrophobic surfaces. The aggregation number of CHAPS is lower than that of other commonly used detergents, which is an important consideration, as the total molecular mass of the protein–detergent complex has a bearing on the overall rotational correlation time and hence on the linewidth of resonances in the NMR spectrum. A disadvantage of CHAPS is that it is not readily available in deuterated form and isotopically labeled protein has to be used since the upfield part of the protein ^1H-NMR spectrum is obliterated by signals from the aliphatic protons of CHAPS. Another disadvantage of nondeuteration of CHAPS is that magnetization can potentially be transferred via spin diffusion and chemical exchange processes (Wüthrich, 1986) providing extra relaxation pathways which may ultimately lead to broader spectral lines.

The addition of CHAPS (17 nM) to solutions of V_H increased the thermal stability of the V_H monomer, with no evidence of precipitation at 30°C during the NMR experiment, and allowed the acquisition of better-quality spectra. The improvement in spectral quality upon addition of CHAPS should enable the structure of this V_H domain to be determined using NMR.

The expression in *E. coli* and purification of milligram amounts of the V_H domain of anti-neuraminidase antibody NC41 provide an opportunity to determine the structure of this single antibody domain in solution and to compare the structure with that determined for NC41 Fab by X-ray crystallography (Tulip *et al.*, 1992). V_H domains with and without a hydrophilic C-terminal peptide marker (FLAG) were expressed and purified, and the V_H domain without the additional FLAG residues was chosen for NMR studies, as the FLAG peptide did not influence the properties (such as solubility) of the V_H domains. Circular

dichroic spectra indicated that the V_H solubilized in 2 M GuHCl had the same structural features as soluble V_H isolated without denaturant.

The NC41 V_H domain at moderate protein concentrations (1–3 mg/ml) at 4°C showed little propensity to dimerize; however, V_H dimers formed over a period of several days at 25°C at 5–10 mg/ml during the collection of NMR spectral data resulting in poor spectral quality. Attempts to improve the solubility of V_H domains either by substituting hydrophobic with hydrophilic residues (in our case with Trp→Glu at 110) or by substituting camelid consensus residues (Davies and Riechmann, 1994; Muyldermans *et al.*, 1994) may significantly alter the structural integrity of V_H domains, particularly at the V_H/V_L interface. Detergents such as CHAPS, *N*-octylglucoside, and ethylene glycol are better alternatives to prevent this dimerization and were shown to produce improved spectral quality to allow the collection of data which should enable the structure of the NC41 V_H domain to be determined.

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