Folding of an antibody variable domain in two functional conformations in vitro: calorimetric and spectroscopic study of the anti-ferritin antibody VL domain

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Understanding refolding pathways of recombinant antibody fragments is essential for efficient production of these proteins of high biomedical significance. The recombinant VL domain of mouse anti-human ferritin antibody F11 formed two distinct functional conformations obtained by refolding from bacterial inclusion bodies using two different procedures. Involvement of a dialysis step at pH 2–3 resulted in the VL-1 conformation with fluorescence of the highly conserved Trp-35 residue quenched by the spatially proximal disulfide bond. This conformation was identical to the ‘native’ VL domain folded in host cells and purified from the cytoplasm. In the absence of the acidic dialysis step, the VL domain adopted a previously unreported conformation, VL-2, that demonstrated prominent fluorescence due to a local structural disorder around Trp-35. Furthermore, VL-2 showed changes in secondary structure and significantly lower stability as determined by differential scanning calorimetry and denaturant-induced unfolding. While more flexible VL-2 binds human ferritin both in solution and after surface adsorption of the antibody domain, the VL-1 conformer needs an adsorption-induced conformational change to allow the access of ferritin to the antigen-binding site. Noteworthy, the two macroscopic conformations constitute kinetically trapped dimers and do not interconvert at elevated temperatures (3 weeks at 37°C or 15 min at 60°C), which indicates a high energetic barrier between them. As a major finding, this paper provides the first description for two stable and functional conformations of an antibody domain.

Keywords: conformational stability/conformers of folding/protein refolding/recombinant antibody/VL domain

Introduction

More than a decade ago, a limited number of proteins were found to exist in more than one stable conformation under physiological conditions, which challenged the thermodynamic hypothesis of protein folding proposed by Christian Anfinsen (Anfinsen, 1973). The best known examples are serine proteases (α-lytic protease and subtilisin) (Zhu et al., 1989; Baker et al., 1992), the serpin family of protease inhibitors (Franke et al., 1990; Stein and Chothia, 1991; Devlin and Bottomley, 2005) and luciferase (Sinclair et al., 1992). Alternative conformations of a protein may significantly differ in local and non-local interactions, thermodynamic stability and activity.

A few multi-domain proteins from the immunoglobulin family, including lymphocyte cell adhesion protein CD2 (Murray et al., 1995) and λ1-type human light chain Loc (Huang et al., 1996), adopted multiple compact structures as demonstrated by X-ray crystallography. In the latter case, three distinct spatial structures were obtained at different ionic strengths and pHs of the crystallization solution. CD2 is capable of folding into two equilibrated but unevenly populated conformations, with the less populated state representing a metastable dimer formed through domain swapping (Murray et al., 1995). Conformational diversity was also demonstrated for three anti-phenyloxazolone antibodies by bi- and triphasic hapten-binding kinetics that indicated antibody isomerization (Foote and Milstein, 1994). Recently, James and co-authors reported different conformations of the antibody SPE7; these conformations were capable of binding unrelated antigens, as judged from crystal structures and pre-steady-state kinetics (James et al., 2003). In the two studies discussed earlier (Foote and Milstein, 1994; James et al., 2003), the antibody conformers existed in equilibrium and differed due to relatively small rearrangements of the molecule in the antigen binding site.

Recombinant antibody fragments have found manifold applications as universal tools for selective recognition of a large variety of target molecules [see (Hudson, 1998) for a review]. The scFv fragments, which comprise variable domains of light (VL) and heavy (VH) immunoglobulin chains linked through a flexible peptide, represent the smallest entities that retain the antigen-binding site of the full-length antibody (Bird et al., 1988; Huston et al., 1988). Furthermore, several single-domain antibodies comprising either isolated VH or VL domains were shown to preserve biological activity and stability, which makes single-domain antibodies a promising protein design to construct structurally minimal recognition modules of immunotoxins, abzymes and other antibody-derived proteins for therapeutic, diagnostic, and industrial applications (Gavish et al., 1977; Ward et al., 1989; Brinkmann et al., 1993; Davies and Riechmann, 1996; Martsev et al., 2000). Hence, knowledge of the inter-relationships among folding, stability and binding activity of isolated variable domains of immunoglobulins is important for engineering new proteins with clinical and industrial applications. Recombinant antibody fragments are frequently expressed as bacterial inclusion bodies, raising a demand for...
simple and effective refolding procedures that would result in a
homogeneous, thermodynamically stable and functional
macroscopic state (Tsumoto et al., 1998, 2003; Umetsu et al.,
2003). Studies of refolding pathways are therefore essential
for successful production of ‘robust’ recombinant antibodies.

In this paper, we provide the first description of the two
stable and functional conformations of an antibody domain.
We employed the recombinant VL domain derived from the
anti-human ferritin monoclonal antibody F11 (Martsev et al.,
2000; Nymalm et al., 2002). Refolding of the denaturant-
solubilized VL domain from bacterial inclusion bodies using
two distinct protocols resulted in two functional conforma-
tions, VL-1 and VL-2, which possess long-term stability
and do not equilibrate under physiological conditions. Using
differential scanning calorimetry, CD and fluorescence spec-

troscopy, and immunoassays, we demonstrated that VL-1 and
VL-2 differ in the amount of structure, thermodynamic and
pH-stability, and arrangement of the antigen-binding site.
A high degree of structural similarity among variable domains
of light chain of immunoglobulins indicates that refolding of
other recombinant VL domains may result in similar confor-

mational diversity.

Material and methods

Purification of the VL domain

Expression of the VL domain was performed as we described
earlier (Martsev et al., 2000). Inclusion bodies, which con-
tained the major (~95%) fraction of the recombinant
protein, were separated from the soluble fraction of the cell
lysate by centrifugation for 30 min at 35 000 g.

The pellet containing inclusion bodies was solubilized
with 6 M guanidine hydrochloride (Gdn–HCl) for 2 h at
room temperature. After centrifugation for 20 min at
35 000 g, the supernatant was dialyzed against 7 M urea in
25 mM Tris–HCl, pH 7.0 and applied to a Ni\(^2+\)-NTA Sepharose column (Novagen, USA) equilibrated with the
same buffer. After washing the column with 10 volumes of
the same buffer, 0.1 M of imidazole was used to elute the
VL domain. The purity of the VL domain was >95% as
determined by SDS–PAGE. Typically, the overall yield was
~50 mg per 1 l of the cell culture.

To purify the minor cytoplasmic fraction of the VL
domain, the soluble fraction of the cell lysate was dialyzed
against buffer A (0.1 M Tris–HCl, pH 8.1, 200 mM NaCl,
10 mM imidazole). The sample was applied on the
Ni\(^2+\)-NTA-Sepharose column equilibrated with the same
buffer. Bound protein was eluted by increasing concen-
trations of imidazole. The fraction containing the VL
domain was dialyzed against 50 mM sodium phosphate, pH 7.4.

Refolding of the two conformers

The VL domain purified from inclusion bodies was refolded
by stepwise dialysis procedure according to the two refolding
protocols (Fig. 1). To obtain the VL-1 conformer, the refold-
ing-protocol-1 was applied in which the purified VL domain
was sequentially dialyzed against 5, 3 and 1 M urea in 0.1 M
sodium phosphate, pH 2–3, and then the same buffer
without the denaturant. At this stage, some variations in
the refolding buffer composition, pH and ionic strength (Fig. 1,
upper line) did not change the final conformation. The
protein was then dialyzed against 0.1 M Tris, pH 8.1, and,
finally, against 0.1 M sodium phosphate, pH 7.4. To obtain
the VL-2 conformer, we applied the refolding protocol-2
(Fig. 1, bottom line). For this, the VL domain after the
Ni\(^2+\)-NTA chromatography was dialyzed against 5, 3 and
1 M urea in 0.1 M Tris, pH 8.1, or 0.1 M sodium phosphate,
pH 7 and, finally, against 0.1 M sodium phosphate, pH 7.4,
without the denaturant.

Molecular mass determination

Gel filtration chromatography was carried out on a Sephacryl
S200 column (1.0 × 106 cm) equilibrated with 50 mM
sodium phosphate, pH 7.4. The following molecular mass
markers were used: IgG (150 kDa); BSA (66 kDa); ovalbumin
(45 kDa); lysozyme (14 kDa). Samples of 0.5 ml containing
0.7–2.0 mg of the VL domain were loaded on the column.
All experiments were performed at room temperature with a
flow rate of 10 ml h\(^{-1}\). Mean values of three independent
experiments are reported.

Interconversions of VL-1 and VL-2

The ability of VL-1 and VL-2 to convert into each other was
studied in two lines of experiments. First, both conformers
were incubated in 0.1 M sodium phosphate, pH 7.4, at
(i) 37°C for 3 weeks or (ii) 60°C for 15 min in a water bath.
Second, urea or Gdn–HCl in 0.1 M sodium phosphate,
pH 7.4, was added to the samples containing VL-1 and VL-2
to final concentrations of 7 M and 6 M, respectively. After incu-
bation for 12 h, the sample of VL-1 was refolded according
to the refolding protocol of VL-2, whereas the sample of
VL-2 was refolded according to the refolding protocol of
VL-1. In both experiments, intrinsic tryptophan fluorescence
was used to monitor the conversions.

CD spectroscopy

CD spectra were recorded on a J-20 spectropolarimeter
(‘Jasco’, Japan) at a protein concentration 0.5–0.7 mg/ml in
50 mM sodium phosphate, pH 7.4, using a thermostated
cuvette with a 1 mm (far-UV CD) or 10 mm (near-UV CD)
quartz cell. Spectra were scanned from 250 to 200 nm
(far-UV CD) or 300 to 250 nm (near-UV CD) at a scan
speed of 5 nm/min. The averaged spectra of three scans were
corrected for the buffer blank.
Fluorescence measurements

All spectra were recorded in triplicates. Intrinsic fluorescence spectra of the proteins were recorded in a 1 cm pathlength quartz cuvette using an SFL-1211 fluorometer (‘Sola’, Minsk, Belarus) at a protein concentration of 0.05 mg/ml in 0.2 M KCl–HCl (pH 1.0–1.8), 0.1 M citrate-phosphate (pH 2.0–6.0), 0.1 M sodium phosphate (pH 6.2–7.8) and 0.1 M Tris–HCl (pH 8.0–10.0). Fluorescence was excited at 280 or 295 nm. Spectra were corrected for the buffer blank.

Fluorescence of protein-bound 8-anilino-1-naphthalene sulfonic acid (ANS) was recorded from 400 to 600 nm after excitation at 360 nm. The protein concentration was 0.05 mg/ml in the above buffers, and the molar ANS-to-protein ratio was 10:1. Spectra were corrected for the ANS fluorescence without a protein.

In equilibrium unfolding experiments, samples of the VL domain (0.025–0.1 mg/ml) in 0.1 M sodium phosphate, pH 7.4, containing varying concentrations of urea, were incubated at room temperature for 24 h. Intrinsic fluorescence excised at 280 or 295 nm was measured at 340 nm using a 1 cm pathlength quartz cuvette. Spectra were corrected for the solutions containing no protein. The baselines in the pre- and post-transition regions were assumed to depend linearly on urea concentration. The data were fitted to a two-state model assuming a linear relationship of the Gibbs free energy change of unfolding, ΔG, and urea concentration, [D]:

\[
y = \frac{y_{0N} + a_N[D] + (y_{0U} + a_U[D]) \exp((m[D] - \Delta G)/RT)}{1 + \exp((m[D] - \Delta G)/RT)},
\]

where \( y \) is the observed signal, \( y_{0N} \) and \( y_{0U} \) and \( a_N \) and \( a_U \) represent intercepts and slopes of native and unfolded baselines, respectively, \( m \) is the slope of the linear dependence of \( \Delta G \) on urea concentration, \( R \) is the universal gas constant and \( T \) is the absolute temperature.

Differential scanning calorimetry

Measurements were performed with a DASM-4 scanning calorimeter (‘Biopribor’, Puschino, Russia), equipped with a computer interface, in a temperature range of 10–80°C at a scan rate of 60 K-h \(^{-1}\). The reference cell was filled with the buffer used to dialyze the protein sample. 0.05–0.1 M sodium phosphate, pH 7.4, was used for the measurements. The heat capacity curves were corrected for the instrumental baseline that was determined with both cells filled with buffer. The protein concentrations varied between 0.5 and 1.8 mg/ml. The averaged heat capacity profiles of five temperature scans were analyzed and deconvoluted using the algorithm of Privalov and Potekhin (1986) and TERCALC software supplied by the DASM-4 manufacturer. Variations of individual measurements for the midpoint transition temperature, \( T_m \), were within 0.4°C.

Determination of antigen-binding activity

Antigen binding affinity was determined in an enzyme-linked immunosorbent assay (ELISA) using the VL domain directly immobilized onto the wells of a polystyrene plate. Increasing amounts of the VL domain (0–1 μg/ml) in 50 mM sodium borate, pH 8.5, were immobilized onto the wells of a polystyrene plate. After 12–16 h of incubation, the wells were washed twice with distilled water. Remaining binding sites on the polystyrene surface were blocked by incubation for 40 min with PBS–BSA, followed by washing. Coated wells were incubated with 1.6 μg/ml of rabbit polyclonal antibodies, raised against the VL domain of the F11 antibody, in 0.2 ml of PBS–BSA for 1.5 h. After two washings, 250 ng of anti-rabbit antibody conjugated with horseradish peroxidase were added in 0.2 ml of PBS–BSA and incubated for 1.5 h. 0.2 ml of 0.02 M o-phenylenediamine and 0.02 M H\(_2\)O\(_2\) in 0.1 M sodium citrate, pH 5, were then added and the solution was incubated with shaking for 5 min. The reaction was stopped by adding 0.05 ml of 10% H\(_2\)SO\(_4\), and the absorbance was determined at 492 nm.

In the direct ELISA, conformers of the VL domain were immobilized by adsorption onto the wells of a polystyrene plate and allowed to react with increasing concentrations of human spleen ferritin. Stock solutions of the VL domain (0.7–0.8 mg/ml) in 50 mM sodium phosphate, pH 7.4, were diluted to 10 μg/ml with sodium borate, pH 8.5, and adsorbed onto the wells of polystyrene plate overnight. The wells were washed with the above buffer, and the remaining binding sites on the polystyrene surface were blocked by incubation for 40 min with PBS–BSA, followed by washing. Coated wells were incubated with increasing amounts of ferritin in 0.2 ml of PBS–BSA. After 2 h and two washings, 200 ng of mouse anti-ferritin monoclonal antibody G10 conjugated with horseradish peroxidase were added in 0.2 ml of PBS–BSA and incubated for 1.5 h. Bound peroxidase activity was measured as above.

Comparison of binding activities of soluble and polystyrene-adsorbed VL-1 and VL-2

To demonstrate the binding capacity of soluble and bound VL domain conformers, an inhibitory assay was constructed. In this assay, the conformers of the VL domain were immobilized by adsorption onto the wells of a polystyrene plate and allowed to compete with increasing concentrations of the soluble VL domain for human spleen ferritin. The VL domain was diluted to 10 μg/ml with sodium borate, pH 8.5, and adsorbed onto the wells of a polystyrene plate overnight. The wells were washed with the above buffer, and remaining binding sites were blocked by incubation for 40 min with PBS–BSA followed by washing. Coated wells were incubated with increasing amounts of ferritin in 0.2 ml of PBS–BSA. After 2 h and two washings, 200 ng of mouse anti-ferritin monoclonal antibody G10 conjugated with horseradish peroxidase were placed into each well. The reaction was stopped by adding 0.05 ml of 10% H\(_2\)SO\(_4\), and the absorbance was determined at 492 nm.

Other methods

Methods for preparation of human spleen ferritin and anti-ferritin monoclonals were reported previously (Martsev et al., 1995; Kravchuk et al., 1998). Native electrophoresis was carried out in a 10% polyacrylamide running gel without a stacking gel. The following buffers were
used: 0.35% acetic acid-NaOH, pH 5, for the running gel; 0.035 M B-alanine-acetic acid, pHi 4.5, as the upper electrode buffer; and 0.1 M sodium acetate, pH 4.5, as the lower electrode buffer. The concentration of the VL domain was determined from the UV absorbance at 278 nm using extinction coefficient of 1.20 mg⁻¹ ml cm⁻¹. Disulfide groups and free thiols were determined in triplicates by the methods of Thannhauser et al. (1984) and Ellman (1959), respectively, in a protein solution containing 3 M guanidine thiocyanate.

Results

Refolding from inclusion bodies

Refolding of the VL domain from 7 M urea by stepwise dialysis according to the two protocols (Fig. 1) resulted in two conformations denoted as VL-1 and VL-2. Each of the two protocols tolerates a small variation in pH, ionic strength and the buffer composition at different stages of refolding. The key difference between the protocols is exposure to low pH (pH 2–3) in refolding protocol-1, which was critical to obtain the formation denoted as VL-1. When exposure to the low pH was excluded from the procedure to give the refolding protocol-2, the stepwise dialysis at near-neutral pH yielded the conformer VL-2. Refolding at slightly acidic pHs (pH 4–5) led to a mixture of VL-1 and VL-2, as judged by intrinsic fluorescence spectra (Supplementary data are available at PEDS online, Figure 1). From these data, we cannot rule out the possibility that our VL domain may refold into more than two conformations. However, we failed to isolate any of them, except for VL-1 and VL-2, using various buffer systems, ionic strengths and pH values during refolding.

VL-1 and VL-2 do not comprise wrong aggregates, non-native disulfides or sulfur derivatives

Refolding from inclusion bodies is a complex process that may be accompanied by multiple off-pathway folding intermediates that constitute misfolded conformations (Clark, 1998; Lilie et al., 1998; Tsumoto et al., 2003). To rule out the contribution of miss-folded species, e.g., the aggregated ones and those with non-native disulfide bridges, to our VL-1 and VL-2 samples, we applied gel permeation chromatography, native electrophoresis, and quantitative analysis of disulfides and free thiols.

The gel filtration chromatography (Fig. 2A) showed that VL-1 and VL-2 are homodimers, which was generally observed for isolated VL domains (Maeda et al., 1976; Gavish et al., 1977; Azuma et al., 1978; Ionescu-Zanetti et al., 1999). Each protein was eluted as a single peak with an apparent molecular mass of 24.8 ± 0.35 kDa (VL-1) or 26.5 ± 0.42 kDa (VL-2). We failed to isolate any oligomers or highly aggregated species from the samples. Likewise, VL-1 and VL-2 migrated as a single band in the native electrophoresis (Fig. 2B).

Determination of the number of disulfide groups under denaturing conditions resulted in 0.97 ± 0.03 and 0.96 ± 0.03 disulfides per protein monomer for VL-1 and VL-2, respectively. This is fully consistent with the two Cys residues that form the single disulfide bridge in the VL domain. The maximal content of free thiols determined by Ellman’s method (Ellman, 1959) did not exceed the value of 0.05 per protein monomer for both VL-1 and VL-2. These measurements strongly suggest that our VL-1 and VL-2 did not comprise appreciable amounts of free thiols and their stable oxygen derivatives. The purification and refolding procedure seems therefore efficient enough for correctly forming the native disulfide bond present in the two alternative conformations of the VL domain. Together, the thiol/disulfide determination and gel permeation chromatography strongly suggest that the samples of VL-1 and VL-2 did not contain considerable amounts of either aggregated species or those with non-native disulfides, nor did they comprise oxidized derivatives of Cys residues.

VL-1 and VL-2 do not equilibrate

From intrinsic fluorescence spectra (see below), we observed no interconversion of the two alternative conformations at room temperature or at 4°C (data not shown). Likewise, incubation in 0.1 M sodium phosphate, pH 7.4, at 37°C for 3 weeks or, alternatively, at 60°C for 15 min, revealed no transition of VL-1 into VL-2 or VL-2 into VL-1 (not shown). Furthermore, we established that neither VL-1 ⇄ VL-2 nor VL-2 ⇄ VL-1 conversion can be induced by adding 7 M urea to VL-1 or VL-2 with subsequent refolding according to the alternative protocol. To successfully convert VL-1 to VL-2 or VL-2 to VL-1 required strongly denaturing conditions (6 M Gdn–HCl) to provide complete unfolding (see Materials and Methods).

Secondary structure and environment of aromatic residues

The far-UV CD spectra of VL-1 and VL-2 at pH 7.4 suggest that the secondary structure is predominantly β-sheet (Fig. 3A), as expected for proteins of the immunoglobulin family. However, the spectrum of VL-1 significantly deviates from that of a typical β-sheet protein due to a prominent contribution from aromatic side chains. Experimental and theoretical studies have demonstrated that aromatic residues clamped in rigid clusters are characterized by a positive CD band in the region 180–220 nm (Bolotina and Lugauskas, 1985; Bolotina, 1987; Manning and Woody, 1989; Chakrabortty et al., 1993) and can therefore strongly influence far-UV CD spectra of proteins (Bychkova et al., 1992;
Uversky and Ptitsyn, 1996; Abramov et al., 2001). The presence of such a band in the difference (VL-1−VL-2) spectrum (Fig. 3A) indicates that the two conformers of the VL domain are different with respect to the environment of aromatic residues. The cluster of aromatic residues that is responsible for the distorted far-UV CD spectrum of VL-1 may be less rigid or absent in VL-2.

The near-UV CD spectra of VL-1 and VL-2 are distinct from that of the denatured VL domain, indicating asymmetric environment of aromatic residues, i.e. the presence of a tertiary structure (Fig. 3B). However, the spectrum of VL-1 is clearly different from the spectrum of VL-2, which is another indication of alterations in the arrangement of aromatic residues.

**Tertiary structure**

The intrinsic fluorescence spectra of both VL-1 and VL-2 suggest compact conformations with aromatic fluorophores protected from quenching by solvent, as judged from the location of the emission maxima (Fig. 4). Unfolding of the VL domain with 6 M Gdn–HCl leads to exposure of the single and highly conserved tryptophan residue (Trp-35) to the polar environment of the solvent, thus inducing a red shift in the maximum of the fluorescence spectrum (Fig. 4A and B). A compact structure of the conformers was confirmed by the lack of fluorescence emission of the bound hydrophobic dye ANS (Fig. 5), the generally recognized probe for partially folded protein conformations. Furthermore, the intrinsic fluorescence spectra of VL-1 are distinct from those observed for VL-2. On excitation at 280 nm, the spectrum of VL-1 exhibited a single emission maximum at about 305 nm, which corresponds to the fluorescence of Tyr residues in proteins devoid of Trp fluorophores (Fig. 4A). Excitation at 295 nm resulted in only background level of emission of Trp-35 (Fig. 4B). This indicates that the fluorescence of Trp-35 is strongly quenched by the disulfide bond which is the commonly observed internal quencher of tryptophan fluorescence in isolated VL domains (Ohage and Steipe, 1999; Martsev et al., 2002; Ewert et al., 2003). In contrast to the intrinsic fluorescence spectra of VL-1, which are similar to those obtained for many other VL domains, VL-2 demonstrated prominent tryptophan emission with the maximum at around 338 nm (Fig. 4B). This, in combination with the correctly linked disulfide bond, is a strong indicator of an altered environment of Trp-35 in the VL-2 conformation. Furthermore, intrinsic fluorescence studies suggest that VL-1 did not contain considerable amounts of VL-2, as the emission of the single tryptophan of VL-1 is almost completely quenched.

**pH-stability**

The two VL conformers demonstrated distinct pH-stability. On lowering pH from 8 to 2, the environment of Trp-35 within VL-1 remained unaltered, as judged by the Trp fluorescence that remained completely quenched over this pH range (Fig. 5A). Likewise, binding of the hydrophobic dye ANS was on the background level between pH 8 and 2 (Fig. 5B). A sharp increase in the Trp-35 fluorescence of VL-1 was observed below pH 2 and accompanied by enhanced binding of ANS (Fig. 5B), thus indicating partial unfolding of the tertiary structure with concomitantly removing the Trp residue from the vicinity of the internal quencher. In contrast to VL-1, the intrinsic fluorescence of VL-2 did not significantly change between pH 8 and pH 4, and demonstrated a biphasic change between pH 4 and pH 2 with only about 1.5-fold increase of fluorescence intensity at pH 1.5 compared to pH 8 (Fig. 5A). In ANS binding studies (Fig. 5B), the VL-2 conformer demonstrated a broad transition with low cooperativity over the pH range 2–8, thus indicating gradually increased binding of ANS due to the exposure of hydrophobic clusters to the solvent. The complicated pH-induced unfolding curves obtained for VL-2 indicate that unfolding might occur through formation of intermediate partially structured states. This was confirmed by the analysis of the intrinsic fluorescence spectra using the ‘phase diagram’ approach (Kuznetsova et al., 2004), which revealed two unfolding intermediates of VL-2 formed at pH 2.3–2.5 and pH 4–4.5 versus no intermediates in the case of VL-1 (Supplementary data are available at PEDS online, Figure 3). Together, the results of pH-induced unfolding studies indicated lower pH-stability of tertiary structure for the VL-2 conformer.

**Fig. 3.** (A) Far-UV CD spectra of the two conformers of the VL domain. The dash-and-dotted curve represents the difference spectrum of VL-1 and VL-2. (B) Near-UV CD spectra of VL-1 and VL-2. The spectra were recorded at a protein concentration 0.6 mg/ml in 50 mM sodium phosphate, pH 7.4, at room temperature. Representative spectra are shown.
Thermodynamic stability

Our calorimetric measurements revealed that thermodynamic stability of both VL-1 and VL-2 is consistent with stability of fully compact proteins. Thermal unfolding of VL-1 and VL-2 resulted in a sharp heat absorption peak (Fig. 6A), as previously observed for many globular proteins. Repetitive cycles of heating and cooling revealed a variable extent of reversibility depending on the end temperature of the scan and the buffer pH and composition (data not shown). The maximal reversibility at pH 7.4 was ~80% for VL-1 and ~60% for VL-2. This makes the calorimetric profiles of the two conformers amenable to equilibrium thermodynamic analysis (Privalov and Potekhin, 1986). The VL-1 conformer demonstrated higher stability in terms of the midpoint transition temperature (62 versus 59°C observed for VL-2) and transition enthalpy, $\Delta h$ (22.2 ± 1.1 J·g$^{-1}$ versus 14.7 ± 0.7 J·g$^{-1}$). The heat absorption peak obtained for each of the two conformations was well approximated by a single two-state transition (Fig. 6A, inset). The approximation data, together with clearly seen differences in $T_m$ and $\Delta h$, strongly suggest that VL-1 and VL-2 each constitutes a major macroscopic state of the VL domain, with contamination by the alternative conformer being less than 10%.

To compare thermodynamic stability of VL-1 and VL-2 at room temperature in terms of the Gibbs free energy change on unfolding, $\Delta G$, we analyzed urea-induced unfolding curves monitored by intrinsic fluorescence (Fig. 6B). Both VL-1 and VL-2 showed reversible unfolding, and the transition curves did not change on changing the protein concentrations (data not shown). The experimental data were well fit by the equation based on the two-state approximation (see Materials and Methods). Consistent with the calorimetric data, the VL-1 conformer demonstrated higher thermodynamic stability in terms of $\Delta G$ (30.7 ± 1.6 versus 24.5 ± 1.9 kJ·mole$^{-1}$ for VL-2). The $m$ values calculated for the two conformers (7.6 ± 0.4 kJ·mole$^{-1}$·M$^{-1}$ for VL-1 and 5.1 ± 0.5 kJ·mole$^{-1}$·M$^{-1}$ for VL-2) were typical of two-state transitions induced by urea (Myers et al., 1995). Phase diagram method (Kuznetsova et al., 2004) showed no

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Fig. 4. Intrinsic fluorescence spectra of the two conformers of the VL domain. Fluorescence was excited at 280 nm (A) and 295 nm (B). The protein concentration was 0.05 mg/ml. The buffer was 50 mM sodium phosphate. Representative spectra are shown.

Fig. 5. Distinct pH-stability of VL-1 and VL-2. pH-induced unfolding was monitored by intrinsic fluorescence at 340 nm (A) and fluorescence of protein-bound ANS at 470 nm (B). Excitation: 295 nm for intrinsic fluorescence and 360 nm for ANS fluorescence. See Materials and Methods for details.
intermediates in urea-induced unfolding of VL-1 and VL-2 (data not shown).

Binding to the antigen

The ferritin-binding capacities of the two conformations were first compared by the direct binding assay with the VL domain immobilized by adsorption onto the wells of a polystyrene plate (Fig. 7). To provide reliable estimates, we showed that VL-1 and VL-2 demonstrate the same level of adsorption on the polystyrene surface, as determined with rabbit polyclonal antibodies raised against the VL domain (Fig. 7A). Despite differences in folding and stability of VL-1 and VL-2, the antigen binding curves of the adsorbed VL domain (Fig. 7B) yielded similar affinity constants for the VL-1 and VL-2 conformers ($2.8 \pm 0.4 \times 10^7$ M$^{-1}$ for VL-1 and $1.2 \pm 0.2 \times 10^7$ M$^{-1}$ for VL-2, respectively). Given that presumed differences in the spatial arrangement of the antigen-binding site in VL-1 and VL-2 might be lost or partially hidden due to adsorption-induced conformational changes shown for many proteins (Friguet et al., 1984; Jemmerson, 1987), we additionally considered functionality of the two VL conformers in the inhibitory immunoassay (Fig. 8) that allows antigen-binding measurements in solution. In the soluble immunoassay system, less stable VL-2 was capable of efficiently binding to the antigen irrespective of whether VL-1 (Fig. 8A) or VL-2 (Fig. 8B) was used to immobilize ferritin. In contrast, the more stable VL-1 conformer demonstrated significantly lower ferritin-binding capacity in the two formats of the soluble immunoassay (Fig. 8A and B). These results strongly suggest that differences in spatial arrangement of the binding sites of VL-1 and VL-2 do exist.

Comparison with the cytoplasmic VL domain

A minor fraction of the VL domain containing less than 5% of the total recombinant protein was expressed into the cytoplasm of Escherichia coli cells (data not shown). Using Ni$^{2+}$-NTA chromatography without denaturants, we purified this cytoplasmic VL domain that was folded into soluble and...
stable conformation in vivo, and compared it with the VL-1 and VL-2 forms that were refolded in vitro from inclusion bodies. In the gel permeation chromatography, intrinsic fluorescence and calorimetric studies, the properties of the cytoplasmic VL domain were virtually identical to those obtained for the VL-1 conformer (intrinsic fluorescence spectra are shown in Figure 2, Supplementary data are available at PEDS online). These data suggest that the conformation and stability of our VL-1 obtained by in vitro refolding protocol-1 was identical to that produced in the minimal amount in vivo by expression in E. coli.

Discussion

The VL domain of antibody F11 refolds in two distinct macroscopic states

When refolded from insoluble inclusion bodies, the VL domain of the mouse antibody F11 adopted two alternative conformations, VL-1 or VL-2, depending on the refolding protocol. Importantly, each of the two conformations constituted the major macroscopic state of the VL domain attainable under the respective refolding protocol. Furthermore, the prominent structural and functional differences between the two alternative forms did not result from contributions from unfolded and aggregated species as well as those with non-native disulphide bridges, free thiols or their oxygen derivatives.

The most evident discriminatory feature of the VL-1 conformer is the environment of Trp-35, which is distinct from that of VL-2, as demonstrated by fluorescence spectroscopy. As commonly observed for VL domains (Ewert et al., 2003), in VL-1 the emission of Trp-35 is quenched due to close proximity to an intramolecular quencher which is generally thought to be the disulfide bond (Ohage and Steipe, 1999; Ewert et al., 2003). In contrast, the prominent tryptophan fluorescence of VL-2 with the maximum at 338 nm seems to be a unique feature that indicates an altered local conformation in the environment of the single Trp residue.

The key difference between the two refolding protocols is the exposure to the low pH (pH 2–3) in refolding protocol-1. Additional studies are needed to reveal the exact mechanisms that lead to formation of either of the two conformations; this work is underway. One possible explanation is compensation of the positive net charge of the VL domain (pI ~ 9) by chloride anions on lowering pH with HCl down to pH 2. The effect of charge compensation by various anions was shown to significantly change the conformation of many proteins (Goto et al., 1990). This compensation of electrostatic repulsion forces in protocol-1 might allow formation of the rigid cluster of aromatic residues detected by CD in VL-1 (Fig. 3A), whereas uncompensated positive charges in refolding protocol-2 might interfere with hydrophobic interactions of the residues constituting the cluster and lead to formation of the alternative environment of Trp-35 in VL-2.

Environment of Trp-35 strongly contributes to folding and stability of the VL domain

Our fluorescence and calorimetric measurements are consistent with a hypothesis that the rigid cluster formed by a number of aromatic residues of the VL domain, including Trp-35, strongly stabilizes the structure of VL-1. This cluster, which we call a ‘conformational lock’, is absent or distorted in VL-2. Indeed, interactions forming the ‘conformational lock’ in VL-1 are remarkably stable between pH 8 and 2 and might therefore be attributed to predominantly hydrophobic forces. This suggestion is consistent with the crystal structure of the VL-1 conformer [Protein Data Bank (PDB) ID 1F6L, (Nymalm et al., 2002)]. In this structure, Trp-35 is surrounded by hydrophobic and aromatic amino acids and is within 5 Å from the disulfide bridge (Supplementary data are available at PEDS online, Figure 4). The cluster around Trp-35 becomes compact on in vitro refolding using protocol-1 as early as at pH 2 in the absence of urea.
conformers of folding have been reported, e.g. conformations. Several other examples of kinetically trapped in Fig. 9 illustrates the free energy landscape for the two genetic barrier that prevents interconversion. The scheme given that the two VL conformations are separated by a high energetic barrier according to the respective protocols. Therefore, we conclude consequently refolding into either VL-1 or VL-2 conformation.

VL-1 denaturant (up to 7 M urea). No appreciable conversion of elevated temperature (15 min at 60°C or 2 weeks at 37°C) or 2 M or 2 weeks at 37°C) or lytic protease (Baker et al., 1992), subtilisin (Zhu et al., 1989) and β subunit of luciferase (Sinclair et al., 1994). In these cases, however, formation of the alternative non-functional conformation resulted from either proteolytically cleaving the pro-region (α-lytic protease, subtilisin) prior to (re)folding, or from lacking the α subunit (luciferase) during the folding process.

Functional activity of VL-1 and VL-2

The two alternative conformations of the VL domain are capable of binding human spleen ferritin with closely similar affinities, as determined by ELISA with the VL conformers immobilized by adsorption on the polystyrene surface (Fig. 7). However, the binding affinities of the two conformers were distinct when the assay comprised soluble VL conformers (Fig. 8). In contrast to the more ‘flexible’ VL-2 form, VL-1 in solution showed a dramatic decrease in ferritin-binding capacity. Thus, the VL-1 conformer required surface adsorption to exert its binding activity. One could presume that adsorption-induced conformational changes of VL-1 allowed the binding site to fit the structure of the antigenic epitope on ferritin. The difference in antigen-binding activity between the two conformers is not unexpected given the environment of Trp-35 is involved in formation of the pro-region (α-lytic protease, subtilisin) prior to (re)folding, or from lacking the α subunit (luciferase) during the folding process.

Biological significance of the VL-2 conformer

Whether VL-2 or other alternative VL conformations can be formed in vivo remains to be established. One essential note in this regard is that VL domains are probably not evolutionally optimized for folding in the absence of the partner domains, CL and VH. Furthermore, in vitro refolding protocols involve

The VL-1 and VL-2 conformers are kinetically trapped

A distinctive feature of the major part of protein conformers described thus far is their ability to equilibrate under physiological conditions or at least convert into the alternative state, in particular at elevated temperatures or at moderate concentrations of a denaturant. This is the case for several antibodies (Foote and Milstein, 1994; Murray et al., 1995; James et al., 2003) and other proteins that demonstrate conformational diversity, e.g. rabbit muscle adenylate kinase (Zhang et al., 1998), RNase A and bovine seminal RNase (Giancola et al., 2000; Sorrentino et al., 2000). In contrast to these previous studies, we could not detect interconversion for the two VL conformations using transient exposure to elevated temperature (15 min at 60°C or 2 weeks at 37°C) or denaturant (up to 7 M urea). No appreciable conversion of VL-1 → VL-2 or VL-2 → VL-1 type occurred unless the conformers were fully unfolded in 6 M Gdn–HCl with subsequent refolding into either VL-1 or VL-2 conformation according to the respective protocols. Therefore, we conclude that the two VL conformations are separated by a high energetic barrier that prevents interconversion. The scheme given in Fig. 9 illustrates the free energy landscape for the two conformations. Several other examples of kinetically trapped conformers of folding have been reported, e.g. α-lytic protease (Baker et al., 1992), subtilisin (Zhu et al., 1989) and β subunit of luciferase (Sinclair et al., 1994). In these cases, however, formation of the alternative non-functional conformation resulted from either proteolytically cleaving the pro-region (α-lytic protease, subtilisin) prior to (re)folding, or from lacking the α subunit (luciferase) during the folding process.

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conditions that are not likely to occur in vivo. In particular, in vitro refolding of proteins extracted from inclusion bodies excludes interactions with intracellular chaperones. In the recent years, however, the growing use of truncated recombinant antibodies for medical and industrial applications has brought into attention possible pathways of refolding of these non-natural proteins. Antibody VL domains are known to share a high degree of structural similarity. Importantly, both Trp-35 and its environment are highly conserved in other VL domains (e.g. see 11VL, 1KIR, 1MAJ structures from PDB and references (Constantine et al., 1994; Essen and Skerra, 1994; Fields et al., 1996)). Therefore, one cannot exclude the possibility that other isolated VL domains could exist in macroscopic conformations similar to VL-1 and VL-2 when refolded from a denatured state, e.g. in large scale industrial purification of proteins solubilized from inclusion bodies. The high antigen-binding activity of VL-2 in combination with simplicity of the purification procedure makes this conformer a good candidate for biomedical and biotechnological use. Recently, we constructed and characterized a potential immunotoxin VL-barnase that comprises the VL domain of antibody F11 and barnase, a bacterial RNAse (Martsev et al., 2004; Tsybovsky et al., 2004). Comparison of thermal unfolding profiles and calorimetric enthalpies of VL-barnase and conformers of the VL domain indicates that the VL domain within the fusion protein folds into the VL-2 rather than VL-1 conformation, which supports the hypothesis that alternative functional conformations of recombinant proteins may be of significant biomedical and industrial importance.

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References