Partially structured state of the functional VH domain of the mouse anti-ferritin antibody F11

Sergey P. Martsev[a,*], Anatoly P. Dubnovitsky[a], Oleg A. Stremovsky[b], Alexander A. Chumanevich[a], Yaroslav I. Tsybovsky[a], Zinaida I. Kravchuk[a], Sergey M. Deyev[b,c]

[a] *Institute of Bio-Organic Chemistry, National Academy of Sciences of Belarus, Minsk 220141, Byelorussia*
[b] *Shemyakin and Ovchinnikov Institute of Bio-Organic Chemistry, Moscow, Russia*
[c] *Institute of Gene Biology, Russian Academy of Sciences, Moscow, Russia*

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Abstract An antibody combining site generally involves the two variable domains, VH from the heavy and VL from the light chain. We expressed the individual VH domain of the mouse anti-human ferritin monoclonal antibody F11. The loss of affinity was not dramatic ($K_a = 4.0\times10^7$ M^{-1} versus 8.6×10^8 M^{-1} for the parent antibody) and comparable to that previously observed for other VHs. However, the functional VH domain adopted a partially structured state with a significant amount of distorted secondary and compact yet greatly destabilized tertiary structures, as demonstrated by spectroscopic and calorimetric probes. These data provide the first description for a functional antibody domain that meets all the criteria of a partially structured state. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: VH domain; Antigen-binding; Antibody folding and stability

1. Introduction

The antigen-binding site in most antibody molecules is composed of the non-covalently linked variable domains, VH from the heavy chain and VL from the light chain. Recombinant VH domains were recently reported to provide the specific binding of the antigen in the absence of a partner VL domain [1–3]. Accordingly, VH domains are considered prime candidates for designing minimal recognition modules of immunotoxins, abzymes and other antibody-derived recombinant proteins for therapeutic, diagnostic and industrial applications [4–6]. The discovery in *Camelidae* (camel and llama) of structurally unique antibodies lacking the light chain and therefore generating a high-affinity binding site without the assistance of VL domains [7] stimulated a rapid progress in overcoming the two major drawbacks of VH domains, their limited solubility and lower affinity [2,3,6,8].

The VH domains were shown, by nuclear magnetic resonance and X-ray crystallography, to adopt a typical immunoglobulin fold with two adjacent disulfide-linked β-sheets [9–15]. These data suggest a completely folded conformation for the VH domains. However, the antigen-binding site spans a significant part of the VH molecule, and folding and stability of antibody variable domains were shown to be dependent on the structure of the complementarity-determining regions (CDR loops) [16–19]. Therefore, one can presume distinct correlates between folding, stability and affinity in isolated variable domains, and knowledge of these correlates is critical for further engineering of single-domain recognition modules. In support of this presumption, spectroscopically determined thermal stability was shown to be high for two out of six llama VHs [20], while for other previously reported VHs thermal stability varied in a wide range [3,20,21]. Thermodynamic stability, as a most reliable quantity characterizing protein folding in solution, was not determined for VH domains by the high-resolution method of differential scanning calorimetry. In this work, we applied a combination of calorimetric and spectroscopic probes to the VH domain derived from the anti-human ferritin monoclonal antibody F11 (mouse IgG2a/κ subclass) and provide here the first description of a functional antibody domain that does not attain complete folding under the physiological conditions and constitutes a partially unfolded, molten globule-like state.

2. Materials and methods

2.1. DNA cloning procedures

A fragment encoding the VH domain was generated by PCR from the gene scFv (single-chain Fv fragment)-F11 that encoded the VH-linker-VL construct (single-chain antibody F11 [22]). The plasmid pETscF11 was used together with the following PCR primers: GGATCCCATATGCAGGTGCAGCTGAAGCAG (forward); GCCTGGCTCGAGTGAGGAGACTGTGAG (reverse). The resulting PCR fragment was digested with the restriction enzymes *Nde*I and *Xho*I and gel-purified for subsequent ligation into the T7 expression vector pET-22b+ which includes a sequence for a C-terminal hexahistidine tag immediately downstream of the *Xho*I site. The ligation mixture was used to transform *Escherichia coli* XL1-Blue cells, and the nucleotide sequence of the resulting construct obtained from a single colony was confirmed by dideoxy sequencing.

2.2. Expression of VH domain

E. coli BL21(DE3) cells transformed with the pET22VH plasmid were grown in 1 l of LB broth containing ampicillin (100 mg/l) at

*Corresponding author. Fax: (375)-172-648761.
E-mail address: martsev@iboch.bas-net.by (S.P. Martsev).

Abbreviations: ANS, 8-anilino-1-naphthalene sulfonic acid; CDR, complementarity-determining regions; GdnHCl, guanidine hydrochloride; K_a, association/affinity constant; PMSF, phenylmethanesulfonyl fluoride; scFv, single-chain Fv fragment; VH, antibody heavy chain variable domain; VL, antibody light chain variable domain

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37°C until the culture reached the middle of the log phase. Then, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 20 μM and the bacterial cells were grown for an additional 4 h at 37°C. The cells were harvested by centrifugation at 3000×*g* for 10 min, suspended in 60–80 ml of 50 mM Tris–HCl, pH 8.0, containing 0.15 M NaCl and 1 mM phenylmethanesulfonyl fluoride (PMSF), centrifuged as above and washed twice with the same buffer. The cells were resuspended in 60–80 ml of the above buffer, disintegrated by sonication and centrifuged at 15 000×*g* for 30 min. The sediment of inclusion bodies typically contained VH domain with a purity of 30–40%.

2.3. Purification and refolding procedures

The pellet of inclusion bodies containing the VH domain was solubilized with 6 M guanidine hydrochloride (GdnHCl) in buffer A (0.05 M sodium borate, pH 8.5) for 1 h at room temperature. After centrifugation for 45 min at 40 000×*g*, the supernatant was dialyzed against 6 M urea in buffer A containing 1 mM PMSF, then centrifuged to remove precipitated material and loaded on a Ni-NTA-Sepharose column equilibrated with buffer A without imidazole. The column was washed with 10 volumes of the buffer, and the bound VH domain was eluted with buffer A containing 250 mM imidazole. The fractions containing the VH domain were pooled and dialyzed against buffer A, then loaded on the DEAE-cellulose column equilibrated with buffer A. Under these conditions, the VH domain did not bind to the column, in contrast to residual contaminating proteins. To refold the purified VH domain, the flowthrough fraction collected after ion exchange chromatography was dialyzed against 3 M, then 1 M urea in buffer A, with centrifugation after dialyses performed as above. Finally, the protein was dialyzed against 0.05 M sodium borate buffer, pH 8.5, centrifuged, and used for further experiments. Typically, the yield of refolded and purified VH domain was 20–40 mg from 1 l of the cell culture. Purification of the monoclonal anti-ferritin antibody F11 [23,24] and human spleen ferritin [23,25] was described previously. The VL domain of the F11 antibody was expressed in inclusion bodies and purified according to the previously reported procedure [26].

2.4. Determination of antigen-binding affinity

The antigen-binding affinity was determined in a competition enzyme immunoassay as described earlier [22]. In the assay procedure, binding of the biotinylated antibody F11 to immobilized human spleen ferritin was quantified in the presence of variable concentrations of the VH domain or, alternatively, the VL domain or parent F11 antibody, as a competitor. The assay was performed at room temperature in triplicates. Variations in the K_a determination were within 25%.

2.5. Fluorescence measurements

Fluorescence spectra of the proteins and protein–8-anilino-1-naphthalene sulfonic acid (ANS) complexes were recorded at room temperature with a protein concentration of 0.05 mg/ml in a 1 cm path length cuvette on a SFL-1211 fluorometer (Solar, Belarus) as we previously described in detail [27]. The protein tryptophan fluorescence was excited at 295 nm, and the ANS fluorescence was excited at 360 nm. The ANS to protein molar ratios were equal to 10 and 120 for the VH domain and parent F11 antibody, respectively.

2.6. Circular dichroism (CD)

Far-UV CD spectra were recorded on a J-20 spectropolarimeter (Jasco, Japan) at a protein concentration of 0.3–0.6 mg/ml as previously described [26]. Mean residue ellipticities were calculated using the value of 115 for the mean residue weight.

2.7. Differential scanning calorimetry

Measurements were performed with a DASM-4 scanning calorimeter (Biopribor, Pushchino, Russia) in the temperature range 10–100°C at a scan speed of 60 K/h as described previously [26]. The protein concentrations ranged between 0.5 and 1.5 mg/ml. Heat capacity curves were analyzed using software TERMCALC and WSCAL supplied by the DASM-4 manufacturer.

2.8. Size-exclusion chromatography

The protein oligomerization state was determined by size-exclusion chromatography at room temperature using a BIO-SIL SEC 250-5 column (0.78×30 cm) and Pharmacia high-performance liquid chromatography (HPLC) system, which was calibrated with molecular mass standards (thyroglobulin, 650 kDa; IgG, 150 kDa; ovalbumin, 45 kDa; myoglobin, 17.8 kDa; B_{12}, 1.34 kDa). The sample volume was 0.1 ml, and the protein concentration was 1 mg/ml.

2.9. Quantitative assay for disulfides and free thiols

The number of disulfides in the purified and refolded VH domain was determined by the method of Thannhauser et al. [28] in a protein solution containing 3 M guanidine thiocyanate, a denaturing agent that does not interfere with the assay. Free thiols were assayed with 5,5′-dithio-bis(2-nitrobenzoic acid) as described by Ellman [29] both in the presence and absence of the denaturant.

2.10. Other methods

SDS–PAGE was performed according to Laemmli [30] and stained with Coomassie brilliant blue R-250. Concentrations of proteins were determined from the absorption of a 0.1% solution in a 1 cm path length cuvette at 280 nm. The absorption values of 1.62 for the F11 antibody, 1.15 for the VL, and 2.28 for the VH domains were calculated from the known amino acid sequences according to [31].

3. Results

3.1. Monomeric state and antigen-binding affinity of the VH domain

Quantitation of disulfides and free thiols for the VH domain obtained by our refolding/purification procedure showed that VH did not contain refolding conformers derived from incorrect disulfide bonding or formation of sulfur derivatives. In four different preparations of VH, the number of disulfides was 0.97 ± 0.05, and no free thiols were detected, which is fully

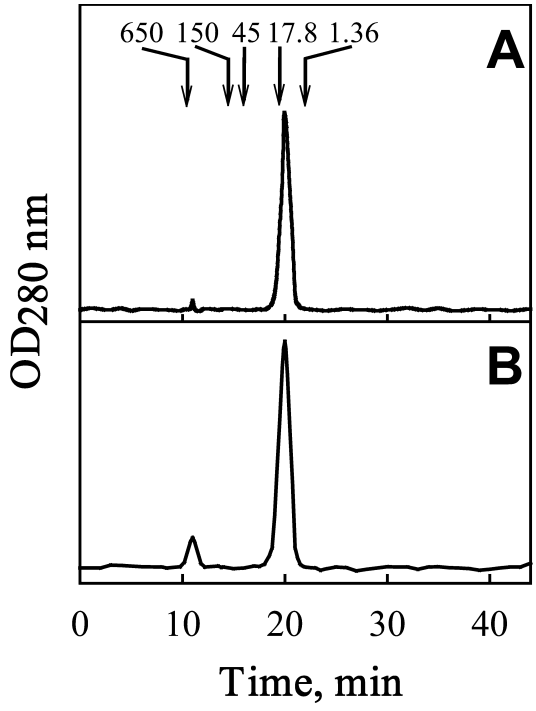

Fig. 1. Size-exclusion HPLC of the VH domain on a BIO-SIL SEC 250-5 column (0.78×30 cm). Samples of VH were applied on the column either immediately after refolding and concentrating procedure (A), or after 4 days of storage at 2–4°C (B). Elution buffer: 0.05 M borate, pH 8.5. Protein concentration: 0.6 mg/ml. Arrows indicate elution time of the molecular mass standards that were as follows: 650 kDa, thyroglobulin; 150 kDa, rabbit immunoglobulin G; 45 kDa, ovalbumin; 17.8 kDa, myoglobin; 1.36, B_{12} vitamin.

consistent with a single disulfide bond present in the VH domain. Previously, a similar purification/refolding procedure yielded correct disulfide bonding for the VL domain and VH-linker-VL construct (the scFv fragment) derived from the same F11 antibody [22,26].

The predominantly monomeric state of the VH domain, consistent with the previous data [1,32,33], was demonstrated by size-exclusion chromatography (Fig. 1). We observed a sharp and symmetric peak corresponding to an apparent M_r of ~15 kDa, as expected from the known amino acid sequence (13 991 Da) and SDS–PAGE data. After 4 days of storage at 4°C, a small amount (less than 10%) of soluble higher oligomers with apparent M_r exceeding 200 kDa was observed. Therefore, freshly prepared protein samples were used for our physical and functional measurements to avoid uncertainties resulting from any small fraction of oligomeric VH.

The VH domain displayed ~23 times lower ferritin-binding affinity (Fig. 2) than the parent full-length F11 antibody ($K_a = 4.0\times10^7$ M^{-1} and 8.6×10^8 M^{-1}, respectively). This difference in the affinity constants most probably reflects the contribution of the partner VL domain to generating the high-affinity antigen-binding site. The VL domain displayed ~45-fold decrease in binding affinity versus the parent antibody F11 (Fig. 2), thus indicating a greater contribution of the VH domain to the binding affinity. A similar decrease in the antigen-binding affinity has previously been observed when isolated VH domains were compared with their parent full-length antibodies [1], and the same order of magnitude for the affinity constant has been obtained for a series of VH domains [33]. Thus, the decrease in the binding affinity of our VH domain is not dramatic and allows reliably detectable binding to the specific antigen, human spleen ferritin. Our affinity measurements confirm the prediction that VH domains may constitute minimal binding modules that are capable of specifically recognizing antigens and can be used as targeting moieties for engineered immunotoxins and other fusions of biomedical significance. Given the reduction in affinity, not all of three hypervariable CDR loops in our VH domain might be involved in binding of ferritin. It was recently shown that the camel anti-carbonic anhydrase VH domain interacts with the antigen with nanomolar affinity through the surface-exposed third hypervariable loop without involvement of the CDR1 and CDR2 loops [15].

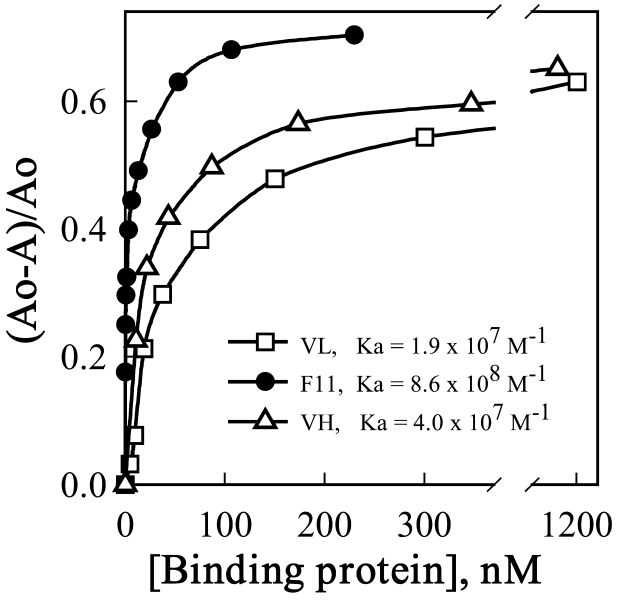

Fig. 2. Antigen-binding affinity of VH domain, VL domain and full-length parent antibody F11 determined by competitive ELISA. The K_a values were obtained from double-reciprocal plots. A and A_0 – absorbance at 492 nm, respectively in the presence or absence of a competitor.

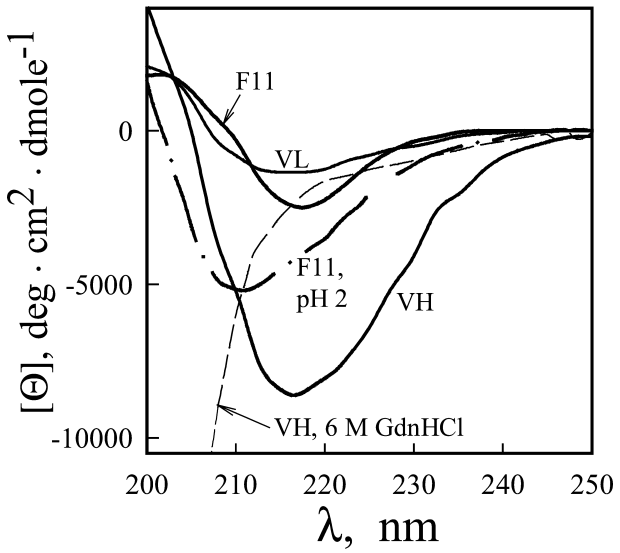

Fig. 3. Far-UV CD spectra of the VH domain, parent full-length antibody F11 and its VL domain.

3.2. CD

Far-UV CD spectrum of the VH domain (Fig. 3) demonstrates a prominent band at 216 nm with a ~three-fold increase in negative ellipticity versus the native full-length antibody F11 or its isolated VL domain. This spectrum is also distinct from that of denaturant-unfolded VH. A similar increase of negative ellipticity in the far-UV region has been observed at strongly acidic pH for partially unfolded states of the mouse monoclonal antibodies and their Fab fragments [34–36], as well as the isolated CH3 [37] and VL [38,39] domains. Together, these data and our CD spectra indicate that the anti-ferritin VH domain adopts a non-native secondary conformation with a significant amount of a β-sheet structure which is markedly distorted and resembles the structure of immunoglobulins partially unfolded under strongly acidic conditions.

3.3. Fluorescence spectroscopy

The intrinsic fluorescence spectrum of the VH domain (Fig. 4A) suggests a compact conformation with aromatic fluorophores protected from quenching by solvent, as judged by the emission maximum around 338 nm in the native protein and a red shift to 352 nm observed after unfolding in the denaturant. However, the VH domain is capable of binding to the hydrophobic dye ANS (Fig. 4B). Binding of ANS represents a generally acceptable probe for a partially unfolded tertiary structure that allows the access to a protein hydrophobic core [40,41]. Neither the denaturant-unfolded VH domain

nor the native F11 antibody and its VL domain bind the hydrophobic dye. Noteworthy is that a partially structured state obtained at pH 2 for the parent F11 antibody [26] and for the VL domain (Fig. 4B) displayed pronounced binding of ANS similar to that observed for the VH domain at neutral pH. Taken together, the fluorescence spectroscopy data are indicative of the partially unfolded tertiary conformation of the VH domain with significantly destabilized tertiary interactions. The fact that the isolated VL domain does not bind ANS at neutral pH demonstrates that the high ANS-binding propensity of our VH domain cannot be attributed to solvent-exposed hydrophobic residues which are buried in full-length antibodies within the domain interfaces.

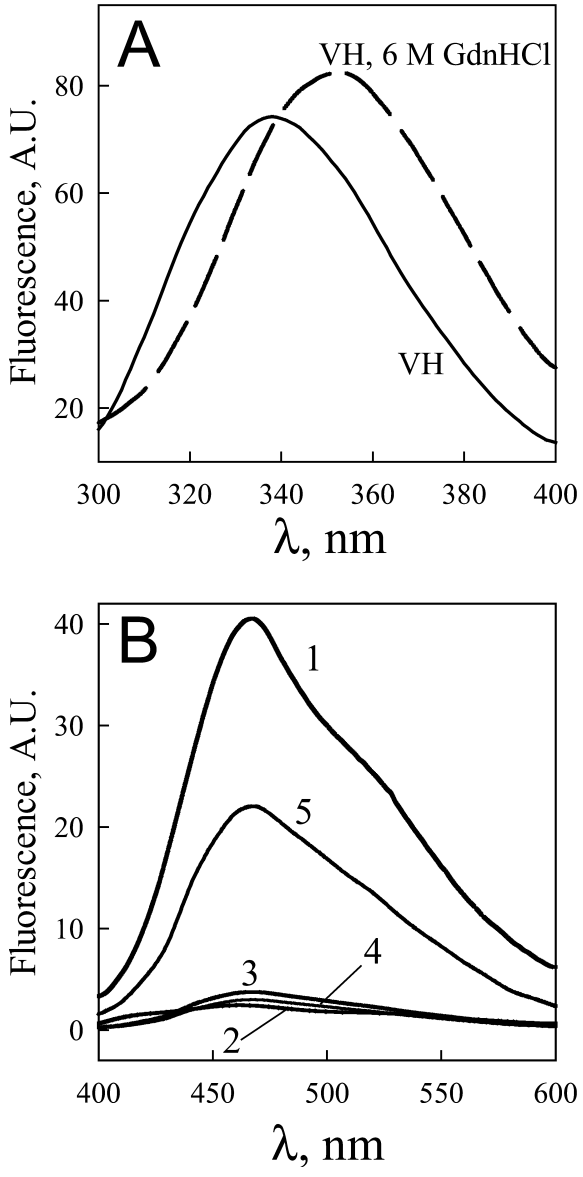

Fig. 4. A: Intrinsic fluorescence spectra of the VH domain. B: Fluorescence spectra of a hydrophobic probe, ANS, in the presence of the following proteins: 1, 'native' VH domain; 2, unfolded VH domain (6 M GdnHCl); 3, native parent antibody F11; 4, native VL domain; 5, partially unfolded VL domain (pH 2).

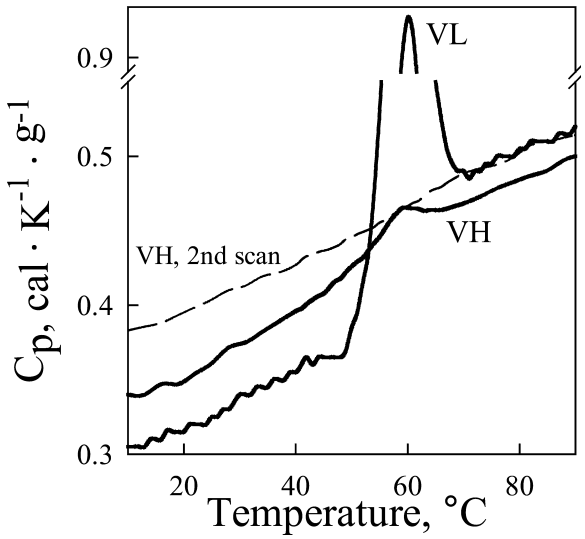

Fig. 5. Differential scanning calorimetry of the VH domain in comparison with the VL domain under the optimized solvent conditions. Buffers: 0.05 M sodium phosphate, pH 7.0 for VL and 0.05 M sodium borate, pH 8.5 for the VH domain.

3.4. Thermodynamic stability

Differential scanning calorimetry revealed a low-cooperative and irreversible thermal transition of the VH domain. When compared to the isolated anti-ferritin VL domain, which is completely folded [26], the tertiary structure of the VH domain is quite distinct (Fig. 5). First, the VH domain does not display the sharp heat absorption peak as the VL domain does. Although the difference in midpoint transition temperature, T_m, determined for the two proteins is rather small (57°C for VH and 60.5°C for VL), the transition enthalpy for the VH domain is dramatically low ($\sim$0.15 cal/g versus 3.9 cal/g). Second, the heat capacity of the VH domain in the pre-transition region (below 50°C) is markedly higher, suggesting partial exposure of hydrophobic residues, consistent with the ANS-binding data. However, the heat capacity does not reach the value characteristic for fully unfolded VH, as demonstrated by a comparison of the first and second calorimetric scans. This comparison indicates the presence in the VH domain of a tertiary structure with a fraction of hydrophobic amino acids involved in hydrophobic packing. Thus, our calorimetric study strongly suggests that the VH domain possesses a compact tertiary conformation that is, however, significantly destabilized and constitutes a partially structured state.

4. Discussion

In this work, we demonstrate that the functional VH domain of the mouse anti-ferritin antibody F11 does not attain complete folding under physiological conditions. Instead, it adopts a partially structured state with a significant amount of a distorted secondary structure and compact yet greatly destabilized tertiary structure. These data provide the first description for a functional antibody domain that meets all the criteria of a partially structured state highly populated under physiological conditions. In this regard, our VH domain can be attributed to the recently described family of

'natively unfolded' proteins that adopt a partially structured conformation under physiological conditions [42,43]. For some of these proteins, intrinsically disordered conformations adopt a fully folded structure upon binding to their biological target [43]. Our results therefore rise the question as to whether the F11-reactive epitope of ferritin can be involved in inducing complete folding of the antibody variable domain; this question, however, cannot be resolved within the scope of the current study.

Given the typical immunoglobulin fold previously reported for the recombinant VH domains [6,33], the structural origin of incomplete folding shown for our VH domain cannot be attributed solely to stabilizing *trans* and *cis* interactions between the VH and partner VL and CH1 domains that are present in Fab fragments and naturally occurring antibodies but lacking in the isolated VHs. It seems more reasonable to suggest that a unique sequence of CDR loops in our anti-ferritin VH domain might constitute a major determinant of its partially unfolded conformation. This suggestion is consistent with the previously reported critical contribution of CDR loops to folding and stability of variable domains [16–19]. An alternative explanation that would involve a strongly destabilizing contribution of the C-terminal hexahistidine tag seems unlikely because a large number of engineered proteins are known to tolerate this tag without major conformational alterations. Furthermore, the VL domain of the same F11 antibody did reach complete folding and functionality with the C-terminal six histidines [26]. To provide direct evidence for a critical contribution of the CDR loops into the partially unfolded conformation of our VH, thermodynamic stability studies of engineered VHs with variable CDR loops grafted into the same framework region are required.

Previously, we demonstrated that the isolated VL domain of the F11 antibody is functional and does reach complete folding, in contrast to the functional VH-linker-VL fusion protein (scFv fragment F11) that comprises the two domains trapped in a partially unfolded state [22,26]. Plückthun and co-workers provided convincing evidence that premature interactions between partially structured VH and VL domains within an scFv fragment might result in a kinetic trap along the folding pathway [44,45]. Given these data, a partially unfolded conformation for both VL and VH domains within the scFv fragment F11 under physiological conditions can be attributed to premature interactions that the partially structured VH domain establishes with the partner VL domain before the latter attains complete folding. Additional studies are needed in order to establish whether the combination of functionality and partially folded conformation is unique for our VH domain or it can be also observed for other variable domains.

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References

[1] Ward, E.S., Gussow, D., Griffiths, A.D., Jones, P.T. and Winter, G. (1989) Nature 341, 544–546.
[2] Davies, J. and Riechmann, L. (1995) Bio/Technology 13, 475–479.
[3] Davies, J. and Reichmann, L. (1996) Protein Eng. 9, 531–537.
[4] Scheriff, S. and Constantine, K.L. (1996) Nat. Struct. Biol. 3, 733–736.
[5] Hudson, J.P. (1999) Curr. Opin. Immunol. 11, 548–557.
[6] Hammers-Casterman, C., Atarhouch, T., Muyldermans, S., Robinson, G., Hamers, C., Bajyana Songa, E., Bendahman, N. and Hamers, R. (1993) Nature 363, 446–447.
[7] Ghahroudi, A.M., Desmyter, A., Wyns, L., Hamers, R. and Muyldermans, S. (1997) FEBS Lett. 414, 521–526.
[8] Muyldermans, S., Cambillau, C. and Wyns, L. (2001) Trends Biochem. Sci. 26, 230–235.
[9] Davies, J. and Riechmann, L. (1994) FEBS Lett. 339, 285–290.
[10] Riechmann, L. (1996) J. Mol. Biol. 259, 957–969.
[11] Decanniere, K., Desmyter, A., Lauwereys, M., Ghahroudi, M.A., Muyldermans, S. and Wyns, L. (1999) Structure 7, 361–370.
[12] Spinelli, S., Frenken, L., Bourgeois, D., de Ron, L., Boss, W., Verripss, T., Anguille, C., Cambillau, C. and Tegoni, M. (1996) Nat. Struct. Biol. 3, 752–757.
[13] Spinelli, S., Frenken, L., Hermans, P., Verrips, T., Brown, K., Tegoni, M. and Cambollau, C. (2000) Biochemistry 39, 1217–1222.
[14] Desmyter, A., Transue, T.R., Ghahroudi, M.A., Thi, M.H., Poortmans, F., Hamers, R., Muyldermans, S. and Wyns, L. (1996) Nat. Struct. Biol. 3, 803–811.
[15] Desmyter, A., Decaniere, K., Muyldermans, S. and Wyns, L. (2001) J. Biol. Chem. 276, 26285–26290.
[16] Yasui, H., Ito, W. and Kurosawa, Y. (1994) FEBS Lett. 353, 143–146.
[17] Helms, L. and Wetzel, R. (1995) Protein Sci. 4, 2073–2081.
[18] Hurle, M.R., Helms, L., Li, L. and Wetzel, R. (1994) Proc. Natl. Acad. Sci. USA 91, 5446–5450.
[19] Raffen, R., Stevens, P.W., Boogaard, C., Schiffer, M. and Stevens, F. (1998) Protein Eng. 11, 303–309.
[20] Van der Linden, R.H.J., Frenken, L.G.F., de Geus, B., Harmsen, M.M., Ruuls, R.C., Stock, W., de Ron, L., Wilson, S., Davis, P. and Verrips, C.T. (1999) Biochim. Biophys. Acta 1431, 37–46.
[21] Perez, J.M.J., Renisio, J.G., Prompers, J.J., van Platerink, C.J., Cambillau, C., Darbon, H. and Frenken, L.G. (2001) Biochemistry 40, 74–83.
[22] Martsev, S.P., Kravchuk, Z.I., Chumanevich, A.A., Vlasov, A.P., Dubnovitsky, A.P., Bespalov, I.A., Arosio, P. and Deyev, S.M. (1998) FEBS Lett. 441, 458–462.
[23] Martsev, S.P., Preygerzon, V.A., Mel'nikova, Y.I., Kravchuk, Z.I., Ponomarev, G.V., Lunev, V.E. and Savitsky, A.P. (1995) J. Immunol. Methods 186, 293–304.
[24] Kravchuk, Z.I., Chumanevich, A.A., Vlasov, A.P. and Martsev, S.P. (1998) J. Immunol. Methods 217, 131–141.
[25] Martsev, S.P., Kravchuk, Z.I., Vlasov, A.P. and Lyakhnovich, G.V. (1995) FEBS Lett. 361, 173–175.
[26] Martsev, S.P., Chumanevich, A.A., Vlasov, A.P., Dubnovitsky, A.P., Tsybovsky, Y.I., Deyev, S.M., Cozzi, A. and Arosio, P. (2000) Biochemistry 39, 8047–8057.
[27] Martsev, S.P., Vlasov, A.P. and Arosio, P. (1998) Protein Eng. 11, 377–381.
[28] Thannhauser, T.W., Konishi, Y. and Scheraga, H. (1984) Anal. Biochem. 138, 181–188.
[29] Ellman, G.L. (1959) Arch. Biochem. Biophys. 82, 70–77.
[30] Laemmli, U.K. (1970) Nature 227, 680–685.
[31] Gill, S.C. and von Hippel, P.H. (1989) Anal. Biochem. 182, 319–326.
[32] Reiter, Y., Schuck, P., Boyd, L.F. and Plaksin, D. (1999) J. Mol. Biol. 290, 685–698.
[33] Riechmann, L. and Muyldermans, S. (1999) J. Immunol. Methods 231, 25–38.
[34] Buchner, J., Renner, M., Lilie, H., Hinz, H.-J., Jaenicke, R., Kiefhaber, T. and Rudolf, R. (1991) Biochemistry 30, 6922–6929.
[35] Lilie, H. and Buchner, J. (1995) FEBS Lett. 362, 43–46.
[36] Welfe, K., Misselwitz, R., Hausdorf, G., Hohne, W. and Welfe, H. (1999) Biochim. Biophys. Acta 1431, 120–131.
[37] Thies, M.J.W., Kammermeier, R., Richter, K. and Buchner, J. (2001) J. Mol. Biol. 309, 1077–1085.
[38] Kurana, R., Gillespie, J.R., Talapatra, A., Minert, L.J., Ionescu-Zanetti, C., Carter, S.A. and Fink, A.L. (2001) Biochemistry 40, 3525–3535.

[39] Martsev, S.P., Dubnovitsky, A.P., Vlasov, A.P., Hoshino, M., Hasegawa, K., Naiki, H. and Goto, Y. (2002) Biochemistry 41, 3389–3395.
[40] Semisotnov, G.V., Rodionova, N.A., Razgulyaev, O.I., Uverski, V.N., Gripas, A.F. and Gilmanshin, R.I. (1991) Biopolymers 31, 119–128.
[41] Engelhard, M. and Evans, P.A. (1995) Protein Sci. 4, 1553–1562.
[42] Uversky, V.N., Gillespie, J.R. and Fink, A.L. (2000) Proteins 41, 415–427.
[43] Dyson, H.J. and Wright, P.E. (2002) Curr. Opin. Struct. Biol. 12, 54–60.
[44] Freund, C., Gehring, P., Holak, T.A. and Plückthun, A. (1997) FEBS Lett. 407, 42–46.
[45] Jäger, M. and Plückthun, A. (2000) Protein Sci. 9, 552–563.