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Antiferritin single-chain antibody: a functional protein with incomplete folding?

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Abstract The pET(scF11) plasmid was constructed comprising the gene of a single-chain antibody against human ferritin. This plasmid encodes the leader peptide pelB followed by the heavy chain variable V_H domain, $(Gly_4Ser)_3$ linker peptide, and light chain variable V_L domain. The correctly processed scF11 antibody was expressed in *Escherichia coli* as an insoluble protein without the leader peptide. Purified soluble scF11 was obtained after solubilization in 6 M GdnHCl followed by a sequential dialysis against decreasing urea concentrations and ion-exchange chromatography. ScF11 demonstrated only a ~8-fold decrease in the affinity ($K_a = 5.1 \times 10^8$ M^{-1} in RIA and 1.8×10^8 M^{-1} in ELISA) vs. the parent IgG2a/κ monoclonal antibody F11. The emission maximum of intrinsic fluorescence strongly suggests a compact conformation with tryptophanyl fluorophores buried in the protein interior, consistent with the functionality of the protein. However, scF11 demonstrated (i) the lack of denaturant-induced fluorescence 'dequenching' effect characteristic of the completely folded parent antibody, and (ii) prominent binding, under physiological conditions, of a hydrophobic probe 8-anilino-1-naphthalenesulfonate (ANS) recognizing partially structured states of a protein. These findings are indicative of an incomplete tertiary fold that gives ANS access to the protein hydrophobic core. This work provides the first indication that the functional single-chain antibody scF11 displays some properties of a partially structured state and therefore may possess incomplete folding.

Key words: Single-chain antibody; Human ferritin; Antibody folding and stability; Antigen-binding domain

1. Introduction

Single-chain antibodies, also referred to as scFv proteins, comprise variable domains fused by a linker peptide resulting in the V_H-linker-V_L or V_L-linker-V_H constructs [1,2]. Since introduction of these recombinant antibodies, there has been growing use of scFv proteins as reagents for therapy, diagnostics, and biomedical research. Large-scale production of scFv proteins generally yields insoluble protein aggregates which can be converted to a soluble functional state by the procedure comprising solubilization with GdnHCl and subsequent refolding on removal of the denaturant (see [3,4] for examples). Based on the functionality of scFv proteins, it was generally concluded that they possess the native immunoglobulin fold. However, in comparison with native immunoglobulins and their fragments, single-chain antibodies have lower long-term stability and higher tendency for aggregation [3,5–8]. These observations, which are easier to reconcile with the model of a partially folded rather than completely folded protein, can be considered as an indirect indication of non-native folding of single-chain antibodies. Therefore, it seemed reasonable to study the conformation of an scFv protein using a probe recognizing partially structured protein states.

Hydrophobic dye 8-anilino-1-naphthalenesulfonate (ANS) is the generally accepted probe for proteins with incomplete folding. This probe does not bind to fully folded proteins nor does it interact with completely unfolded polypeptides, but demonstrates pronounced binding to compact partially structured states [9]. Therefore, ANS binding is considered as a reliable indication of a partially disordered three-dimensional structure with fluctuating tertiary interactions allowing the dye to access the hydrophobic core of a protein [10].

In this work, we describe the construction, expression and refolding of the purified single-chain antibody scF11 against human ferritin. Ferritin is a tumor-associated marker for human hepatocellular carcinoma [11], myeloid leukemia and Hodgkin's disease [12,13], and head and neck squamous cell carcinoma [14]. Radiolabeled full-length antibodies to human ferritin were successfully used for therapy of inoperable liver carcinoma and Hodgkin's disease [15]. Produced in *E. coli* in preparative amounts and refolded, the soluble scF11 antibody exhibited a compact and functional conformation which possessed aromatic fluorophores buried in the protein interior. Some folding-related properties of scF11, such as binding activity, solubility, stability, and apparently two-state denaturant-induced unfolding, were similar to those reported for other scFv proteins, except for ANS-binding capacity which was not previously considered. We found that the scF11 antibody demonstrated prominent ANS-binding capacity similar to that of partially structured states. This work provides the first indication that a functional scFv protein displays some properties of a partially structured state and therefore may possess incomplete folding.

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2. Materials and methods

2.1. Enzymes and reagents

Restriction enzymes, *Thermus aquaticus* DNA polymerase, and T7 DNA polymerase were purchased from Fermantas (Lithuania); T4 DNA ligase, *E. coli* DNA polymerase I Klenow fragment, reagent kits for nick-translation, cDNA synthesis, 5′- and 3′-terminal DNA labeling were from Amersham (UK); the reagent kit for PCR was from Perkin Elmer-Cetus (USA), and nutrient media components from Difco (USA). PCR primers CAGGTGCAGCTGAAGCAG, TGAGGAGACTGTGAGTGG, GACATCCAGATGACACAG, CCGTTTTATTTCCAACT, TGACCACACTCCCCTTGGT, GGCCATCGCTGGTTGGGC, GGATCCGCCGCCACCAG were ordered by Fermentas (Lithuania). Labeled reagents: [^{32}P]dATP, [^{32}P]dCTP, and [^{33}P]dATP were purchased from the State Institute of Applied Chemistry (GIPKh, St. Petersburg, Russia). Streptavidin and its conjugate with horseradish peroxidase, PMSF, isopropyl β-D-thiogalactopyranoside, 8-anilino-1-naphthalenesulfonate, guanidine hydrochloride, and bovine serum albumin were from Sigma (USA), and Iodogen was from Pierce (USA). The protein molecular mass markers were from Serva (Germany).

2.2. Bacterial strains

DH 5α (**F**−, Δ(*lac*ZYA*arg*F)U169, *rec*A1, *end*A1, *hsd*R17, (**r-k, m+k**), *sup*E44, Φ80d*lac*ZΔM15, *gyr*A96, *rel*A1, *deo*R, *thi*-1), BL21 (DE3) (**F−, ompT, rb, mb**, λ lysogen containing T7 RNA polymerase under control of lacUV5 promoter).

2.3. DNA cloning procedures

Standard molecular biology techniques were used for constructing the scF11 encoding plasmid [16]. Briefly, DNA fragments were isolated by electroelution into a dialysis sac, and purified by chromatography on NENSORB 20 (NEN-DuPont, USA). Poly(A)$^+$ RNA was isolated from 2×10^7 hybriboma F11 cells producing mouse IgG2a/κ antiferritin antibody and cDNA was synthesized using a random mixture of hexadeoxyribonucleotide primers [16]. Double-stranded cDNA was inserted into pBR322 and the library in *E. coli* was analyzed using probes for mouse γl [17] and κ [18] immunoglobulin genes. The isolated heavy and light chain genes of the F11 antibody were sequenced and used for engineering of the single-chain antibody gene by a PCR-based technique [19]. The complete construct containing the nucleotide sequences encoding the pelB leader peptide, V_H domain, $(Gly_4Ser)_3$ linker peptide, and V_L domain was cloned into the pET3α(x) vector under the control of T7 RNA polymerase promoter [20].

2.4. Expression of scF11

E. coli BL21(DE3)pLysS cells transformed with the pET(scF11) plasmid were grown in 1 l of LB broth containing ampicillin (100 mg/l) and chloramphenicol (170 mg/l) at 37°C until the culture reached the middle of the log phase. After 2.5–3.0 h of growth, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and the bacterial cells were grown for additional 4 h at 37°C. The cells were harvested by centrifugation at $10\,000\times g$ for 10 min, suspended in 60–80 ml of 50 mM Tris-HCl, pH 8.0, containing 0.15 M NaCl and 1 mM phenylmethylsulfonyl 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 $45\,000\times g$ for 20 min. The sediment of inclusion bodies typically contained scF11 with a purity of 50–60%.

2.5. Purification of scF11 and parent F11 antibodies

The pellet of inclusion bodies containing scF11 was solubilized with 6 M guanidine hydrochloride (GdnHCl) in buffer A (0.1 M Tris-HCl, pH 8.0) for 2 h at room temperature. After centrifugation for 45 min at $40\,000\times g$, the supernatant was dialyzed against 5 M urea in buffer A containing 1 mM PMSF, then centrifuged to remove precipitated material and applied on the HiTrapQ anion exchange column (1.7×2.5 cm). Bound proteins were separated by 0–0.5 M gradient of NaCl using Pharmacia (Sweden) FPLC system. The protein fraction eluted between 0.2 and 0.3 M NaCl was collected and 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 phosphate buffer, pH 7.5, centrifuged, and used for further experiments. Typically, the yield of refolded and purified scF11 was 10–20 mg from one liter of the cell culture. The parent mouse monoclonal antiferritin antibody (IgG2a/κ) previously described as antibody HSF102 [21,22] produced by the F11 hybridoma cell line, was purified from ascitic fluid by the procedure of McKiney and Parkinson [23].

2.6. Human spleen ferritin and [^{125}I]ferritin

Human spleen ferritin was isolated as previously described [21,24]. [^{125}I]Ferritin (specific activity 1.5 Ci/g) was obtained by iodination of the protein using Iodogen as an oxidant [22].

2.7. Determination of antigen-binding affinity

The antigen-binding affinity was determined both in a direct radioimmunoassay (RIA) and in a competition enzyme-linked immunosorbent assay (ELISA). The assays were performed at room temperature in triplicates, and the mean values were used for graphic presentation. Variations in K_a determination were within 25%.

Direct binding RIA was performed as described [22] using streptavidin covalently coupled through protein amino groups to polystyrene balls (6.3 mm in diameter, the product of the Institute of Physical Organic Chemistry, Minsk) containing surface-exposed phenaldehyde groups. The balls were incubated in polystyrene tubes with biotinylated F11 or scF11 (2 μg/ball), then washed, and increasing concentrations of [^{125}I]ferritin were added. After 2 h and washing, bound [^{125}I]ferritin was quantitated using a γ-counter (RiaGamma, Wallac, Finland).

In a competition ELISA, polystyrene tubes were coated with rabbit anti-ferritin IgG by overnight incubation with 3 μg of IgG in 0.25 ml of 0.05 M sodium borate, pH 8.5. After 40 min incubation with 1% BSA and washing, 0.5 mg of human spleen ferritin in 0.25 ml of PBS-BSA was added. The tubes were incubated for 1.5 h, then washed twice with distilled water, and 50 ng of biotin-F11 conjugate together with increasing amounts of F11 or scF11 were added to the tubes in 0.25 ml of PBS-BSA. After 2.5 h and two washings, 250 ng of streptavidin-horseradish peroxidase conjugate were added in 0.25 ml of the above buffer. After another 40 min and washings, 0.6 ml of 0.02 M *o*-phenylenediamine and 0.02 M H_2O_2 in 0.1 M sodium citrate, pH 5, was added and incubated with shaking for 10 min. The reaction was stopped by adding 0.2 ml of 10% H_2SO_4, and absorbance was determined at 492 nm.

2.8. Fluorescence measurements

Intrinsic and ANS fluorescence spectra were recorded at 25°C in a 1-cm path length cuvette using SFL-1211 fluorometer (Solar, Belarus) as described in [25]. Samples for fluorescence measurements were prepared by diluting the protein stock solution to a final concentration of 0.1 mg/ml in a buffer with a required denaturant concentration. The protein tryptophan fluorescence was excited at 295 nm. The fraction of scF11 unfolded at increasing denaturant concentrations was calculated according to [3] from the maximum wavelength of intrinsic fluorescence. The ANS fluorescence was excited at 360 nm. The final ANS-to-protein molar ratios were equal to 20 and 120 for the scF11 and parent F11 antibody, respectively, corresponding approximately to the ratio of 10 mol of ANS per 1 mol of an antibody domain. Before recording emission spectra, solutions comprising ANS and the protein were allowed to equilibrate for 2 h at room temperature; in the presence of a denaturant, the time required to reach equilibrium was 16–20 h.

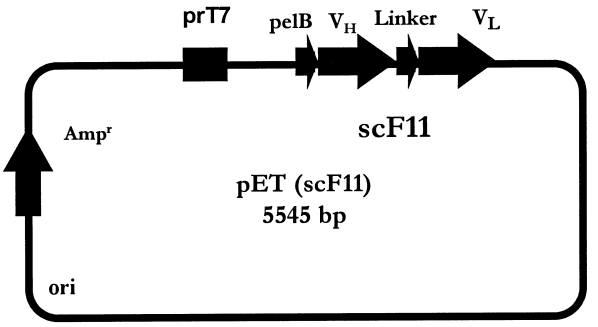
2.9. Other methods

SDS-PAGE was performed according to [26] and stained with Coomassie Brilliant Blue R-250. Concentrations of antibodies were determined from the values of absorption of 0.1% solution in a 1-cm path length cuvette at 280 nm. The absorption values 1.81 for scF11 and 1.62 for F11 were calculated from the known amino acid sequences according to [27]. Automatic Edman sequencing of scF11 was performed with Applied Biosystems (USA) protein sequencer, model 470A.

3. Results

3.1. Construction of the scF11-encoding plasmid

To construct the 'mosaic' gene of the single-chain antibody, the four DNA fragments that encode the leader peptide pelB



1 5 10 15 20 25
MKYLLPTAAAGLLLLAAQPAMAQVQ
LKQSGPGLVQPSQSLSITCTVSGFS
LTSYGVHWVRQSPGKGLEWLGVIWS
GGSTDYNAAFISRLSISKDNSKSQV
FFKMNSLQANDTAIYYCARELVYYF
DYWGQGTTLTVSSGGGGSGGGGSGG
GGSDIQMTQSPASLSASVGETVTIT
CRASENIYSYLAWYQQKQGKSPQLL
VYNAKTLAEGVPSRFSGSGSGTQFS
LKINSLQPEDFGSYYCQHHYGTPFT
FGSGTKLEIKRDPRLIN

Fig. 1. Plasmid pET(scF11) encoding the single-chain antibody scF11 (upper panel) and the amino acid sequence of scF11 (lower panel) deduced from the gene. Amp^r: ampicillin resistance gene; prT7: T7 RNA polymerase promoter; pelB: leader peptide sequence of pectate lyase from *Erwinia carotovora*; V_H and V_L: heavy and light chain variable domain encoding sequences, respectively. Underlined are the pelB leader peptide (double line) and the linker peptide $(G_4S)_3$ (single line).

(which is responsible for the secretion of the protein into the periplasm), the heavy chain variable V_H domain, the linker peptide, and the light chain variable V_L domain of the monoclonal F11 antibody were combined by PCR amplification of the DNA ligase products as described in [19]. The gene was assembled in pUC18 plasmid and at the final stage was inserted into the plasmid pBluescriptsLS(+) containing the universal translation terminator GCTTAATTAATTAAGC. The complete gene (Fig. 1) was included in the plasmid pET3α(x) under the control of T7 RNA polymerase promoter to obtain a system developed by Studier et al. [20] for expression of recombinant genes.

3.2. Expression, purification, refolding

Bacterial expression of scF11 directed by T7 RNA polymerase yielded the amounts of the recombinant protein ranging from 30 to 50 mg per one liter of the cell culture, as estimated from SDS-PAGE. The same method showed the lack of pelB leader sequence in scF11 indicating that the mature protein was produced, which was further confirmed by sequencing the purified protein by automatic Edman degradation. The sequence of 11 N-terminal amino acids, NH_2-Gln-Val-Gln-Leu-Lys-Gln-Val(Ser)-Gly-Pro-Gly-Leu was in full agreement with the sequence deduced from the heavy chain cDNA. The mature antibody was packed in inclusion bodies where scF11 typically constituted 50–60% of the total protein. Solubilization with 6 M GdnHCl and subsequent stepwise dialyses, initially against 5 M urea and then, after ion exchange chromatography, against 3 M and 1 M urea, resulted in a significant loss of contaminating proteins (Fig. 2) due to their lower solubility in comparison with scF11. Also, the HiTrapQ anion exchange chromatography efficiently removed contaminants absorbing at 260 nm, presumably nucleic acids. The final yield of scF11 was 10–20 mg per 1 l of the cell culture with a purity more than 95%.

Correct processing of scF11 also assumes proper bonding of the two expected disulfides; the absence of additional bands during SDS-PAGE without β-mercaptoethanol (Fig. 2, tracks 6 and 7) indicated that there were no scF11 dimers or multimers present in the sample due to formation of intermolecular disulfides. Multi-step refolding by sequential dialysis provided a compact functionally active conformation of scF11 and prevented drastic losses of the protein. In contrast, direct dialysis of the antibody preparation dissolved in 5 M urea against a denaturant-free buffer resulted in a massive precipitation of scF11.

3.3. Binding affinity

The affinity constant, $K_a = 5.1 \times 10^8$ M^{-1}, determined for scF11 in direct binding assay using [^{125}I]ferritin, was only about 8 times purer than K_a of the parent antibody F11 (Fig. 3). Competition ELISA gave approximately 3-fold lower apparent binding affinities for both the parent antibody and scF11 resulting in the same ratio of constants as obtained in RIA.

3.4. Folding of scF11

On decreasing urea concentrations, refolding of purified scF11 resulted in an increasing population of species with Trp fluorophores protected from quenching by the solvent, as judged by a shift in the maximum emission from 349 nm in 8 M urea to 337 nm in the refolding buffer (Fig. 4A, curve 1). This result suggests that the scF11 antibody reached a compact tertiary conformation. However, formation of the

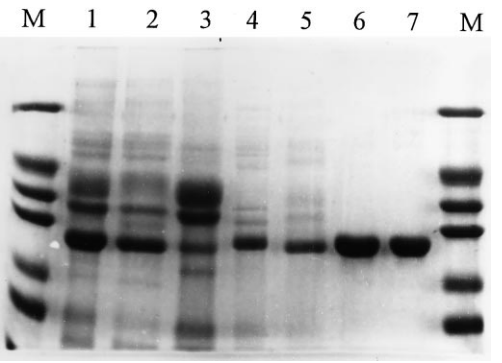


Fig. 2. SDS-PAGE analysis of expression and purification of scF11: Coomassie-stained 15% polyacrylamide gel. Tracks: 1: inclusion bodies solubilized with 6 M GdnHCl; 2, 4, 5: 6 M GdnHCl-solubilized inclusion bodies after dialysis against 5 M, 3 M, and 1 M urea, respectively; 3: the fraction of inclusion body proteins that precipitated during dialysis against 5 M urea; 6, 7: purified scF11 in the presence (6) or absence (7) of β-mercaptoethanol. M: molecular mass markers: 66 kDa, bovine serum albumin; 45 kDa, ovalbumin; 36 kDa, glyceraldehyde-3-phosphate dehydrogenase; 29 kDa, carbonic anhydrase; 24 kDa, trypsinogen; 20 kDa, soybean trypsin inhibitor.

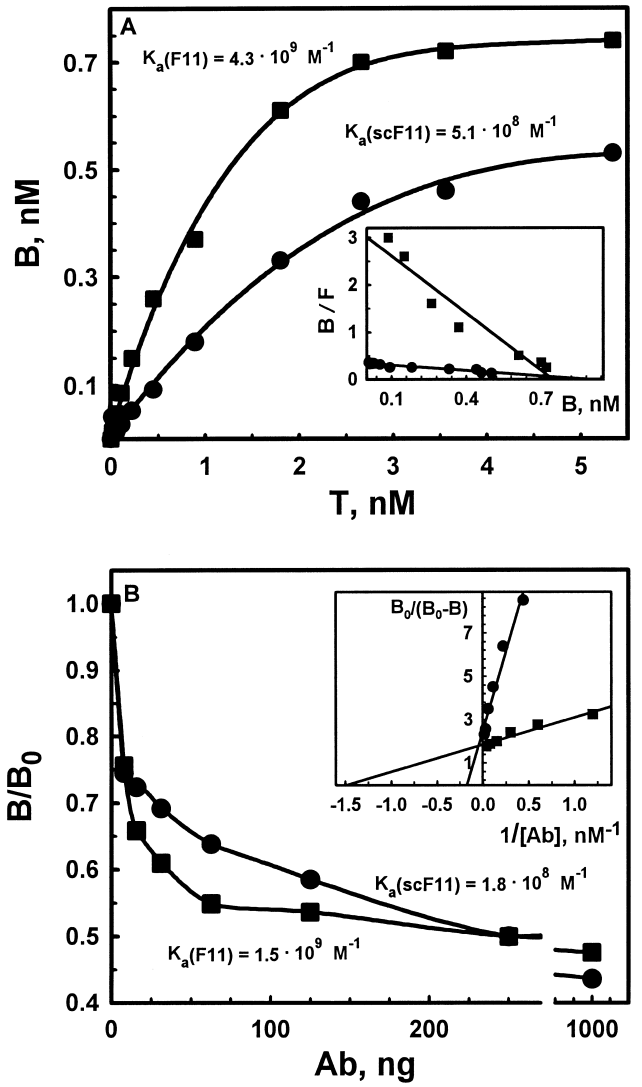


Fig. 3. Antigen binding affinity of scF11 in comparison with the parent antibody as demonstrated by RIA with [^{125}I]ferritin (A) and by ELISA (B). Insets: Scatchard plot (A) and double reciprocal plot (B). B and T in panel A: bound and total [^{125}I]ferritin, respectively. B and B_0 in panel B: bound peroxidase activity in the presence and absence of the competitor, respectively.

final structure of scF11 was accompanied by an increase in binding of a hydrophobic dye, ANS (Fig. 4A, curve 2), which is a general feature of partially structured states of proteins [9,10]. Furthermore, the final structure of scF11 no longer displayed an increase in fluorescence intensity on denaturant-induced unfolding as did the completely folded parent F11 (data not shown). Prominent ANS binding to scF11 observed under physiological conditions was similar to that shown for the partially structured state of the parent F11 antibody at pH 2 (Fig. 4B). This enhanced binding of ANS to scF11 might result from either the exposure of domain interfaces (that are normally masked by partner domains, C_L and C_H1, in full-length antibodies) or a fluctuating tertiary structure characteristic of a partially structured state with non-native tertiary interactions. The first possibility is unlikely since the V_L domain of the same antibody which was expressed, purified, and refolded to a compact functional conformation (Matrsev, Vlasov and Arosio, manuscript in preparation) did not display binding of ANS at pH 7 (data not shown). Thus, one may conclude that high ANS binding results from the incomplete tertiary fold of the scF11 antibody.

In terms of the $[D]_{05}$ values (denaturant concentrations that provide half-maximal unfolding), stability of scF11 (Fig. 4C)

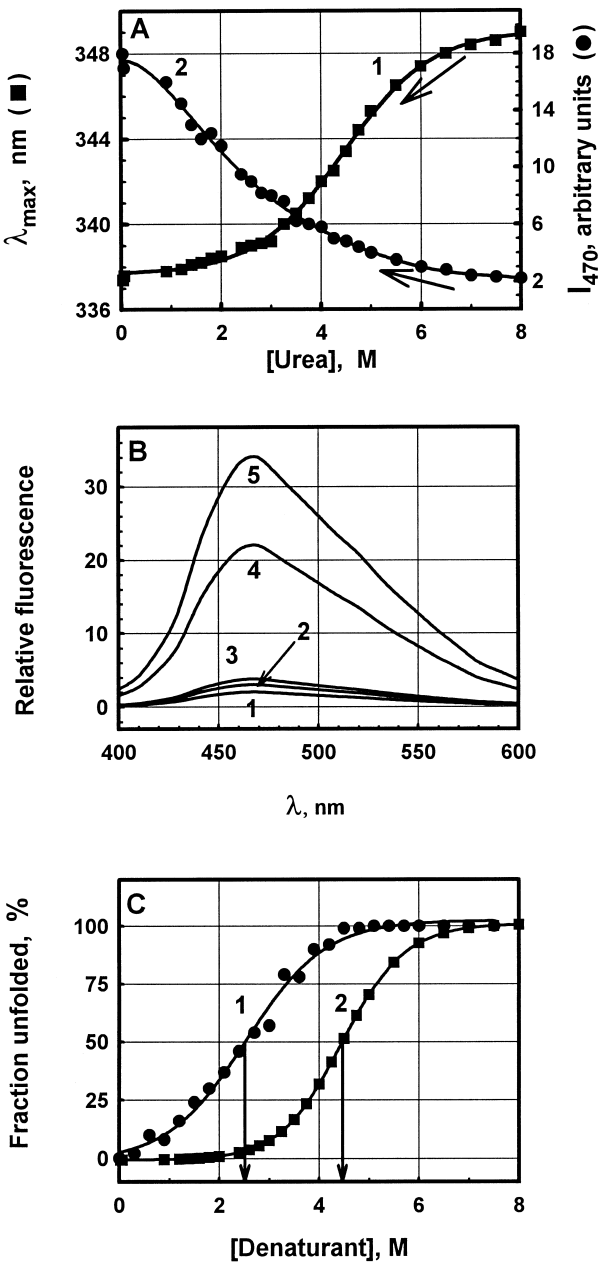


Fig. 4. Fluorescence study of folding and stability of scF11. A: Folding of scF11 monitored by the emission maximum of intrinsic protein fluorescence (1) and by the emission maximum of protein-bound ANS. Stock solution of scF11 (2 mg/ml in 8 M urea) was diluted 100-fold with 0.05 M sodium phosphate, pH 7, containing variable urea concentrations. B: Fluorescence spectra of ANS in the presence of the following proteins: 1, unfolded scF11 (7 M GdnHCl); 2, unfolded F11 (7 M GdnHCl); 3, parent F11 (pH 7); 4, partly unfolded F11 (pH 2); 5, scF11 after refolding (pH 7). C: GdnHCl (1) and urea (2) induced unfolding of scF11 at pH 7. The fraction of the unfolded protein was determined from the maximum wavelength of intrinsic protein fluorescence, measured as a function of the denaturant concentration.

was similar to that previously observed for several scFv proteins. These values for scF11 (4.5 M urea, or 2.5 M GdnHCl, Fig. 4C) are close to or even higher than those reported for other single-chain antibodies (1.0 M GdnHCl or 3.7 M urea [3,28], 4.1 M urea [29], 2.0 M urea [30]). However, scF11 demonstrated low stability against aggregation, which resulted in precipitation of 30–50% of the protein after two weeks at 2–4°C.

4. Discussion

A remarkable feature of the engineered scF11 antibody after its expression, purification and refolding is the ability to bind a hydrophobic dye, ANS, in the physiological solvent conditions. The enhanced ANS-binding capacity of a protein is one of the generally accepted criteria of incomplete folding with fluctuating tertiary structure that allows the dye to gain access to the hydrophobic core [9,10]. Therefore, prominent binding of ANS to the antiferritin scF11 antibody suggests incomplete folding of this protein. Nevertheless, the antigen-binding affinity of scF11 was only ~8-fold lower than that of the parent F11 antibody. This finding indicates that a distorted tertiary structure of scF11 does not destroy the structural integrity of the antigen-combining site. These results partially contradict the generally accepted concept that the protein functionality is tightly associated with a completely folded conformation.

Until now, the ANS-binding capacity of the single-chain antibodies has not been reported, and therefore it remains to be shown whether or not this property is general for scFv proteins. However, there are at least three general features of scF11 and single-chain antibodies described previously, the first of them being a prominent tendency to aggregation. This property may be attributed to either transient exposure of the hydrophobic interior of the protein, or to the linker peptide. The other general features of scFv proteins are their relatively low stability and apparently two-state transition observed on denaturant-induced unfolding. The latter property might equally result from either strong domain interactions with tightly-associated folding of the two domains or, alternatively, from weak, if any, interactions between domains with closely similar transition parameters [3,5,31]. Based on the similarity of features related to folding and stability of single-chain antibodies, one can assume that the antiferritin antibody scF11 is not unique with regard to the observed folding parameters. To verify this assumption, extended folding/stability studies should be carried out using a series of single-chain antibody constructs.

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