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Fusion of Barnase to Antiferritin Antibody F11 VH Domain Results in a Partially Folded Functionally Active Protein

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Abstract—A chimeric protein, VH–barnase, was obtained by fusing the VH domain of anti-human ferritin monoclonal antibody F11 to barnase, a bacterial RNase from *Bacillus amyloliquefaciens*. After refolding from inclusion bodies, the fusion protein formed insoluble aggregates. Off-pathway aggregation was significantly reduced by adding either purified GroEL/GroES chaperones or arginine, with 10-12-fold increase in the yield of the soluble protein. The final protein conformation was identical by calorimetric criteria and CD and fluorescence spectroscopy to that obtained without additives, thus suggesting that VH–barnase structure does not depend on folding conditions. Folding of VH–barnase resulted in a single calorimetrically revealed folding unit, the so-called "calorimetric domain", with conformation consistent with a molten globule that possessed well-defined secondary structure and compact tertiary conformation with partial exposure of hydrophobic patches and low thermodynamic stability. The unique feature of VH–barnase is that, despite the partially unfolded conformation and coupling into a single "calorimetric domain", this immunofusion retained both the antigen-binding and RNase activities that belong to the two heterologous domains.

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Key words: VH domain, barnase, protein folding, immunofusion, molten globule, differential scanning calorimetry

Most globular proteins can adopt partially unfolded conformations known as molten globules. The molten globule is a compact state characterized by well-defined secondary structure and a hydrophobic core that lacks most specific tertiary interactions [1-3]. Most molten globules have been found at low pH or with intermediate concentrations of denaturing and chaotropic compounds. Unusual proteins that are capable of forming the molten globule state under physiological conditions are attributed to the family of intrinsically disordered proteins [4-6]. Some of these proteins can maintain a part of their specific tertiary interactions [1, 7, 8] and also biological activity, a phenomenon that is quite rarely observed [9, 10].

We demonstrated earlier that the VH domain of anti-human ferritin monoclonal antibody F11 forms a conformational state that corresponds to the functionally active molten globule [10]. This conformation is characterized by significantly altered secondary structure and compact but strongly destabilized tertiary structure with exposed hydrophobic sites. The isolated VH domain is a poorly soluble hydrophobic protein that is expressed in *Escherichia coli* cells as inclusion bodies. Fusion of the VH domain of antibody F11 to its natural partner, the isolated VL domain, which is a completely folded, stable, and water soluble protein, via a $(GGGGS)_3$ linker [11] resulted in formation of a two-domain construct (scFv – single-chain variable fragment) that adopted functionally active but partially unfolded conformation typical for molten globules [11, 12]. Scanning calorimetry study showed that in this conformation the two constituting domains form a single calorimetrically revealed structure, the so-called calorimetric domain. During the folding process, early interactions of partially unfolded VH domain with "premature" VL domain in the conforma-

Abbreviations: ANS, 8-anilino-1-naphthalenesulfonate fluorescence dye.

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tion of molten globule stabilize this partially unfolded structure and thus block folding of the whole two-domain scFv fragment.

Fusion of the VL domain with barnase, a bacterial RNase from *Bacillus amyloliquefaciens*, gave a quite distinct result. It was shown that in this case barnase plays the role of an intramolecular chaperone that significantly increases solubility of the VL domain during expression *in vivo* and refolding *in vitro* [13]. The chaperone-like effect of barnase was attributed to specific properties of this protein exhibiting high stability and solubility [14-16] as well as rather small sizes (110 amino acid residues) and compatibility with bacterial expression systems [17].

To elucidate whether barnase can exhibit chaperone-like properties in other chimeric proteins, we fused barnase with poorly soluble and partially unfolded VH domain in the recombinant VH–barnase protein. We suggested that barnase could increase solubility of the VH domain and promote its folding into a more compact conformation. In this study, we have demonstrated that the chimeric protein including the VH domain of antibody F11 and barnase can adopt a partially unfolded state under physiological conditions. The spatial structure of VH–barnase is typical for a protein in a molten globule state that includes a native-like secondary structure and partially unfolded tertiary conformation lacking some stabilizing interactions; the chaperone-like effect of barnase was not found. The unusual feature of VH–barnase is retention of two functional activities, the antigen-binding and RNase ones, within a single calorimetric domain formed by the two heterologous domains, VH and barnase.

MATERIALS AND METHODS

Cloning. The gene encoding the VH domain was amplified from the plasmid pETscF11 [12] using the following primers: 5′-ATATACATATGCAGGTGCAGCTGAAG-3′ and 5′-CATGAGTCGACGGATCCGCCGCCACCAG-3′. The product of the PCR reaction was treated with restrictases *Nde*I and *Sal*I and inserted into the sites *Nde*I-*Sal*I of the plasmid pET (F11-barnase) (unpublished). Besides the insert encoding VH–barnase under control of the T7 RNA polymerase promoter, the resultant plasmid included gene of barstar (protein inhibitor of barnase) under control of its own promoter; this excludes possible toxicity of barnase for expressing cells. Lack of mutations in the VH gene was confirmed by its sequencing.

Expression of VH–barnase. *Escherichia coli* cells BL21(DE3) transformed with the plasmid pET(VH barnase) were incubated at 37°C in LB medium containing 100 μg/ml ampicillin until mid-log phase. After cultivation for 3-4 h, isopropyl-β-D-thiogalactopyranoside (IPTG) (final concentration 1 mM) was added and the cells were incubated for 4 h at 37°C.

The bacterial cells were harvested by centrifugation at 3000*g* for 10 min and then resuspended in 100 ml of 50 mM sodium phosphate buffer, pH 7.4, 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 100 ml of the same buffer, sonicated, and centrifuged at 15,000*g* for 30 min. The insoluble fraction of inclusion bodies contained VH barnase with purity of 20-30%.

Purification of VH–barnase. After sedimentation, inclusion bodies containing VH–barnase were sequentially washed with buffer containing 1, 2, and 3 M urea and incubated at 37°C for 1 h followed by centrifugation at 30,000*g* for 30 min. The inclusion bodies were then dissolved in buffer A (6 M guanidine hydrochloride (GdnHCl), 50 mM sodium phosphate buffer, 5 mM imidazole, pH 7.4) for 1 h at room temperature. The supernatant obtained after centrifugation for 45 min at 30,000*g* was applied to a chromatographic column (1.0 × 5.0 cm) with Ni-NTA-Sepharose (Qiagen, Germany) equilibrated with buffer A. The column was washed with 10 volumes of buffer A, and nonspecifically bound proteins were then eluted by increasing imidazole concentration in buffer A up to 25 mM. VH–barnase was eluted at imidazole concentration in buffer A of 0.25 M. Fractions containing VH–barnase were dialyzed against imidazole-free buffer A.

Expression and purification of GroEL and GroES chaperones. *Escherichia coli* BL21(DE3) cells transformed by the pGro7 plasmid (Takara Bio Inc, Japan) were cultivated in LB medium containing chloramphenicol (20 μg/ml) at 37°C until mid-log phase. After addition of L-arabinose (final concentration of 0.01 M) the cell culture was incubated at 37°C for 4 h. The bacterial cells were harvested by centrifugation at 3000*g* for 10 min and resuspended in 100 ml of 50 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl and 1 mM PMSF. The cells were then centrifuged as above, washed twice with the same buffer, resuspended in it (50 ml), sonicated, and centrifuged at 30,000*g* for 30 min. The resulting supernatant was heated at 60°C for 7 min, cooled, and centrifuged as above. Soluble proteins of the supernatant were precipitated by ammonium sulfate precipitation (75% saturation). After centrifugation at 30,000*g* for 15 min, the precipitate was resuspended in 10 mM sodium phosphate buffer, pH 7.4, containing 0.1 M NaCl, filtered, and applied to a column (2.5 × 100 cm) packed with TSK HW-55f (Toyo Soda, Japan) and eluted with 10 mM sodium phosphate buffer, pH 7.4, containing 0.1 M NaCl. Fractions containing oligomers of GroEL (800 kDa) or GroES (70 kDa) were collected and precipitated with ammonium sulfate (75% saturation). The pellet obtained after centrifugation at 30,000*g* for 15 min was resuspended in 50 mM Tris-HCl, pH 8.6, and dialyzed against the same buffer. Fractions containing GroEL or GroES were applied onto a chromatographic column

(1.5 × 10 cm) containing Toyopearl-DEAE ion exchanger (Toyo Soda), and proteins were eluted by a 0-0.5 M NaCl gradient in 50 mM Tris-HCl, pH 8.6. GroEL and GroES proteins were eluted at 0.3 and 0.25 M NaCl, respectively. The eluate was dialyzed against 50 mM Tris-HCl, 0.1 M NaCl, 0.02% sodium azide, pH 8.1. According to SDS-PAGE and gel-permeation chromatography, purity of both oligomer chaperones was not less than 95%. Chaperone concentration was determined using the Bio-Rad Protein Assay (Bio-Rad, USA).

Refolding of VH–barnase. All experiments on VH–barnase refolding were performed at 4°C. Refolding conditions were optimized by the method of rapid dilution. Denatured protein (6 M GdnHCl, 0.05 M sodium phosphate buffer, pH 7.4) was added dropwise into the refolding buffer (0.05 M sodium phosphate buffer, pH 7.4) under intensive stirring. Refolding buffers contained various additives: 0-10% glycerol, 0-10% sucrose, 0-0.5 M ammonium sulfate, 0-0.5 M NaCl, 0-0.5 M Tris, 0-0.5 M glycine, 0-0.5 M β-alanine, 0-0.05% polyethylene glycol 3550, 0-0.4 M arginine, etc. We also used various buffers for maintenance of pH: 0.05 M citrate-phosphate (pH 4.0-6.0), 0.05 M sodium phosphate (pH 6.2-7.8), and 0.05 M Tris-HCl (pH 8.0-10.0). During refolding based on the stepwise dialysis in the presence of arginine, denatured protein was dialyzed against the same buffer with sequentially decreased GdnHCl concentration. After the end of refolding, the protein solution was centrifuged for separation of precipitated VH–barnase, and soluble protein was determined spectrophotometrically.

Chaperone-assisted refolding. Denatured VH–barnase was dialyzed against 50 mM Tris-HCl, pH 8.1, containing 7 M urea. During the initial stage of refolding, the denatured protein was diluted with 2.5 M urea in the above described denaturant-free buffer containing 10 mM $MgCl_2$ and chaperones GroEL/GroES (proportion with barnase of 1 : 2 : 1, respectively). This protein solution was incubated for 15 min, sequentially dialyzed against 2, 1, and 0.1 M urea (each stage continued for 12 h), and centrifuged at 30,000*g* for 30 min; the supernatant was applied to a chromatographic column (3.0 × 50 cm) containing Sephacryl S200 and the protein was eluted with 50 mM sodium phosphate buffer, pH 7.4; soluble protein was determined spectrophotometrically for calculation of the refolding yield. The protein was concentrated using Amicon filters (Millipore, USA).

Functional activity. *Antigen binding.* Antigen binding activity was determined using an ELISA test system for interaction of human ferritin with the VH domain or VH–barnase adsorbed onto polystyrene. Analyses were performed at room temperature in triplicates, and average values were used in subsequent calculations. Association constants were determined using double reciprocal plots and varying K_a values within 20%. Solution of the VH domain (1 mg/ml) or VH–barnase (1 mg/ml) in 0.05 M sodium-phosphate buffer, pH 7.4, was diluted with 0.05 M borate buffer, pH 8.5, to protein concentration of 10 μg/ml. The solution was then incubated overnight on polystyrene plates. The plates were washed using 0.05 M sodium phosphate buffer, pH 7.4 (washing buffer), and free binding sites on the polystyrene surface were blocked by means of plate incubation with 1% BSA in 0.05 M sodium phosphate buffer and 0.15 M NaCl, pH 7.4 (sodium phosphate buffer-BSA) for 40 min. After washing and subsequent addition of 0.2 ml of sodium phosphate buffer-BSA and increasing amounts of ferritin into wells, the plates were incubated for 2 h. After two washings of the plate wells, 0.2 ml of sodium phosphate buffer-BSA containing 200 ng of the antiferritin monoclonal antibody G10 conjugated with biotin was added. The plates were incubated for 1.5 h, and after washing 0.2 ml of streptavidin–horseradish peroxidase conjugate was added and the plates were incubated for 40 min. After two washings, 0.2 ml of substrate solution (0.02 M *o*-phenylenediamine and 0.02 M H_2O_2 in 0.1 M sodium citrate buffer, pH 5.0) was added, and the plates were incubated for 5 min under vigorous shaking. The reaction was stopped by adding 0.05 ml 10% H_2SO_4, and absorbance was read at 492 nm.

Ribonuclease activity. Quantitative assay of RNA degrading activity of VH–barnase and barnase was performed at 25°C by the method of Kunitz [18]. The decrease in RNA absorbance was measured spectrophotometrically at 300 nm using a cuvette with 1-cm pathlength containing 1 mg/ml yeast RNA in 0.05 M sodium phosphate buffer, pH 7.4.

Fluorescence measurements. Fluorescence spectra of proteins and protein complexes with 8-anilino-1-naphthalenesulfonate (ANS) were recorded at room temperature and protein concentration of 0.05 mg/ml in a cuvette with 10-mm path length using a SFL-1211 or a SM-2202 fluorimeter (both from SOLAR, Belarus) as described in [12]. Protein and ANS fluorescence was excited at 280 and 360 nm, respectively. The molar ratio ANS/protein was 10 : 1 for the VH domain and barnase and 20 : 1 for VH–barnase.

CD spectroscopy. Measurements were performed using a J-20 spectropolarimeter (Jasco, Japan) in a thermostatted cuvette with 1- and 10-mm pathlength in the near- and far-ultraviolet regions, respectively. Protein concentration was 0.3-0.5 mg/ml; the scanning rate was 5 nm/min with time constant of 16 sec. Each spectrum represents an averaged result of three scans. The average value of molecular mass of an amino acid residue used for calculation of molecular ellipticity per residue was 111.7 Da.

Differential scanning calorimetry. Measurements were performed using a DASM-4 scanning calorimeter (Biopribor, Russia) in the range of temperatures from 10 to 100°C at scanning rate of 1 K/min as described by Martsev et al. [11]. Protein concentration was varied from 0.5 to 1.5 mg/ml. Heat capacity functions were analyzed with TERMCALC and WSCAL software from Biopribor.

Quantitative determination of disulfide and thiol groups. The number of disulfide groups of VH–barnase was determined by the Thannhauser method [19] in the presence of 3 M Gdn thiocyanate; this denaturant does not interfere with the analysis. Free thiol groups were determined by the Elman method [20] using 5,5′-dithiobis-(2-nitrobenzoic acid) in the presence and in the absence of the denaturant.

Molecular mass determination. Gel-permeation chromatography of VH–barnase was performed using a Sephacryl S200 column (1.0 × 106 cm) equilibrated with 0.1 M NaCl, 0.05 M sodium phosphate buffer, pH 7.4. The following proteins were used as molecular mass standards: IgG (150 kDa), BSA (66 kDa), ovalbumin (45 kDa), lysozyme (14 kDa). Samples containing 0.25-1 mg of VH–barnase were applied to the column in 0.5 ml. All experiments were performed at 4°C and elution rate of 10 ml/h.

Other methods. Expression and purification of the VH domain were performed as described in [11]. Barnase was obtained from TG1 cells as described in [21]. Ferritin was prepared using a previously described method [10]. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was carried out according to Laemmli [22] and using Coomassie Brilliant Blue R-250 for staining. Protein concentrations were determined spectrophotometrically using specific absorbance coefficients $A^{1\%}_{1cm}$ calculated on the basis of known amino acid sequences of VH–barnase and the VH domain; according to [23] and [24] these were 22.2 and 22.8, respectively.

RESULTS

Expression, purification, and refolding of the VH–barnase immunoconjugate. During expression of VH–barnase in IPTG-induced *Escherichia coli* cells, this protein accumulated as insoluble aggregates within inclusion bodies, and no soluble protein was detected in the cytoplasmic fraction. After solubilization of inclusion bodies with 6 M GdnHCl under non-reducing conditions followed by chromatography of VH–barnase on Ni-NTA-Sepharose under denaturing conditions, the eluted VH–barnase was homogeneous protein with apparent molecular mass of 27 kDa as shown by SDS-PAGE (Fig. 1, lanes *1-3*). This corresponds well to the primary structure of this protein.

Using the methods of Elman and Thannhauser [19, 20], it was shown that purified VH–barnase contains one disulfide bond per molecule and has no free thiol groups. These results are consistent with data that the VH domain of antibody F11 contains two canonic cysteine residues forming a disulfide bond, which has also been found in the VH domains of other antibodies [25], whereas barnase lacks cysteine residues [17]. Additional indication for correct formation of disulfide bonds in VH–barnase is lack of dimeric and multimeric forms of the proteins as shown by SDS-PAGE under non-reducing conditions. The final yield of purified denatured protein varied from 5 to 10 mg per liter of bacterial culture.

During refolding of purified VH–barnase almost all chimeric protein formed insoluble aggregates, and so we performed a series of experiments optimizing refolding conditions (table) and chose two effective refolding systems employing purified GroEL/GroES chaperones or arginine; this resulted in the yield of up to 60% of soluble protein. Preparations of VH–barnase obtained by refolding in the presence of either chaperones or arginine exhibited identical stability and functional activity confirmed by scanning calorimetry and functional analysis. So, in subsequent experiments we used VH–barnase preparations obtained by the arginine refolding as the most available and equally effective method.

Gel-permeation chromatography revealed that renatured VH–barnase is a monomeric protein within three days after its purification. After three days of storage at 4°C, an increase in the fraction of high molecular weight aggregates was observed, and so in subsequent conformational and functional studies we used freshly prepared protein samples.

Differential scanning calorimetry. At neutral pH values, thermal denaturation of VH–barnase is characterized by a single peak of heat absorption with maximum at 44.5°C (midpoint transition temperature, T_m) (Fig. 2, a and b). The presence of the heat absorption peak suggests the presence of a compact protein conformation stabilized by tertiary interactions. Thermal denaturation of VH–barnase at neutral pH was irreversible because after cooling down in the calorimetric cell and subsequent

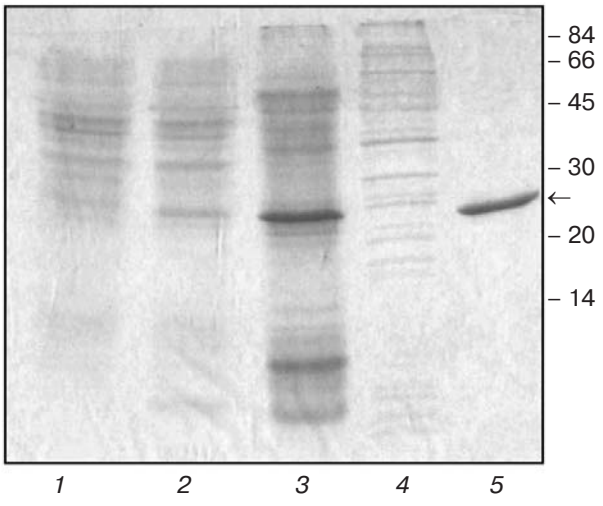

Fig. 1. Expression and purification of VH–barnase: *1*, *2*) cell extract before and after IPTG addition, respectively; *3*) inclusion bodies; *4*) soluble fraction after cell disruption; *5*) purified VH–barnase. Arrow indicates VH–barnase, 27 kDa.

Refolding yield of soluble VH–barnase under various conditions *in vitro*

Additions/methods	Optimal concentration	Refolding yield, %
Without additions	–	5.0
Glycerol	10%	5.5
Sucrose	10%	5.1
Ammonium sulfate	0.5 M	6.0
NaCl	0.5 M	5.0
Tris	0.5 M	5.2
Glycine	0.5 M	5.1
β-Alanine	0.5 M	5.0
Polyethylene glycol 3550	0.05%	5.3
Arginine	0.4 M	60
Gel-permeation chromatography	–	5.0
IMAC chromatography	–	5.0
GroEL-GroES chaperones*	–	50

Note: VH–barnase refolding from the denaturant solution (6 M GdnHCl) was performed by dilution with 0.05 M sodium phosphate buffer, pH 7.4, containing the additions listed in this table or by means of gel-permeation or metal-chelating chromatography. Protein precipitated during refolding was separated by centrifugation, and yield of soluble protein was determined spectrophotometrically. The protein was refolded in the presence of chaperones by dialysis with stepwise decrease in urea concentration (see "Materials and Methods" section).

* Optimal molar ratio GroEL/GroES/VH–barnase was 1 : 2 : 1.

heating of the protein no heat absorption peak was observed (Fig. 2a). Thermal denaturation of many proteins is irreversible at neutral pH generally due to aggregation of unfolded molecules [26, 27]. Formally, accurate calculation of thermal denaturation parameters is possible only for reversible thermal transitions [28]. However, the possibility of enthalpy calculation has also been demonstrated for processes characterized by poor reversibility or lack of reversibility because usually the rate of calorimetric scanning significantly exceeds the rate of the irreversible aggregation process [26, 27, 29]. Therefore, for comparison of thermodynamic stability of tertiary structure of the chimeric protein and its isolated domains we calculated calorimetric enthalpy of denaturation of VH–barnase. The specific calorimetric enthalpy of VH–barnase calculated using the heat absorption curve (Fig. 2b) was ~6 J/g. It should be noted that for most globular proteins with completely folded tertiary structure the values of thermal unfolding enthalpy generally fall within the range 20-30 J/g [28, 30]. The unusually low enthalpy of VH–barnase denaturation suggests loss of a major part of its structural interactions. The midpoint transition temperature (T_m) of the chimeric protein does not coincide with the T_m of its structural modules, the VH domain and barnase. Thermal denaturation of the isolated VH domain of anti-ferritin antibody F11 occurred at 59°C and was characterized by extremely low transition enthalpy of 0.6 J/g (Fig. 2b). This is consistent with previously obtained results and might be explained by a partially disordered conformation of the isolated VH domain [10]. In contrast to the VH domain, isolated barnase is characterized by unusually high (compared with other proteins) transition enthalpy (43 J/g; Fig. 2b) with T_m at 53°C [15, 16, 31]. These data exclude the possibility that the peak of heat absorption of VH–barnase with transition temperature of 44.5°C and enthalpy of 6 J/g (Fig. 2b) could be due to denaturation of native barnase with minimal contribution of partially unfolded VH domain. If this possibility is correct, the expected enthalpy would be significantly higher (20.5 J/g) than the experimentally obtained value.

The presence of the single calorimetrically revealed domain confirms the existence of potent nonspecific interactions between the VH domain and barnase, which prevents independent thermal denaturation of these two domains. Thus, results of scanning calorimetry experiments lead to the following conclusions:

– VH–barnase possesses a compact tertiary structure;

– low thermodynamic and thermal stabilities of the chimeric protein are associated with the loss of a significant part of the tertiary interactions in the structure of each constituting domain;

– there are potent nonspecific interactions between two partially unfolded domains, VH and barnase, which form a single calorimetric domain with parameters of thermal denaturation differing from those obtained for each of the isolated constituents of this protein.

Fluorescence and CD spectroscopy. VH–barnase contains 7 tryptophan and 14 tyrosine residues evenly distributed in the chimeric protein. Therefore, the fluorescence spectroscopy can be used for evaluation of global tertiary structure. On excitation at 280 nm, the fluorescence spectrum of these proteins was characterized by an emission maximum at 366 nm (Fig. 3). This suggests the presence of compact protein conformation protecting aromatic fluorophores against solvent quenching. Compact conformation of VH–barnase is also confirmed by a long-wavelength shift of the emission maximum to 355 nm observed during protein denaturation in 6 M GdnHCl (Fig. 3).

The fluorescent hydrophobic dye ANS is a widely used probe for detection of partially unfolded proteins

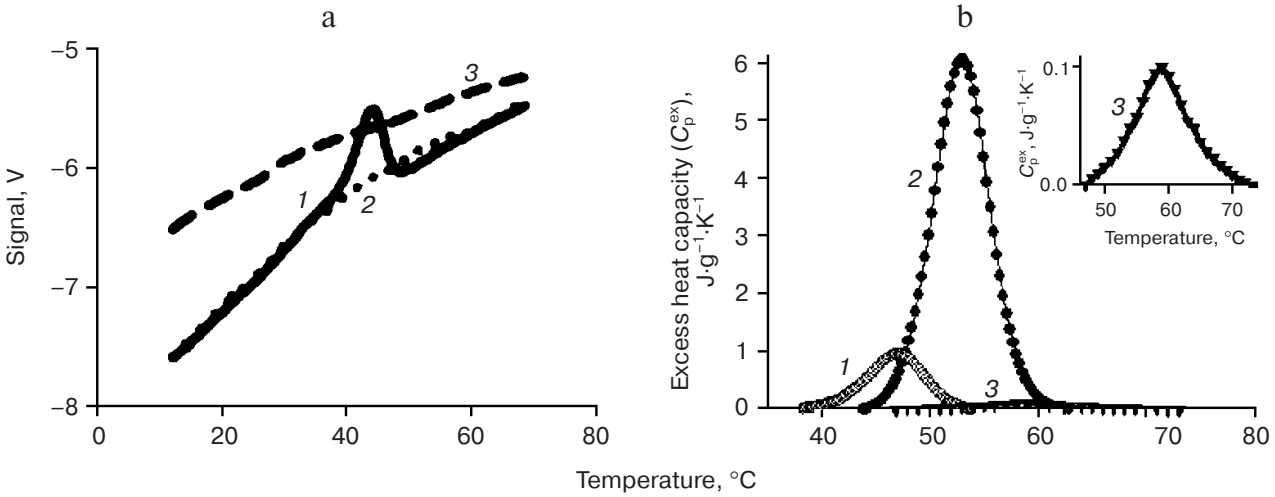

Fig. 2. a) Calorimetric heat capacity curves for VH–barnase in 0.05 M sodium phosphate buffer, pH 7.4: *1*) first heating; *2*) second heating; *3*) baseline. b) Excess heat capacity curves of protein in 0.05 M sodium phosphate buffer, pH 7.4, for VH–barnase (*1*), barnase (*2*), and the VH domain (inset, *3*).

[32-34]. ANS binds to solvent-exposed hydrophobic sites of partially unfolded proteins, which result in a marked short-wavelength shift and significant increase in fluorescence emission. Addition of ANS to fully unfolded or completely compact proteins is not accompanied by changes in fluorescence parameters [35]. It was shown that under physiological conditions VH–barnase binds ANS, and this is accompanied by a corresponding change in fluorescence parameters (Fig. 3, inset), thus confirming exposure of hydrophobic sites. These results are consistent with the unusually low enthalpy of denaturation of VH–barnase.

In the far-ultraviolet region, the circular dichroism spectrum of VH–barnase demonstrates a negative extremum at 218 nm and a shoulder at 230 nm (Fig. 4a). This is typical for β-sheet secondary structures with significant contribution of aromatic amino acids. This result is consistent with the β-sheet structure of the isolated VH domain [10] and known predominantly β-sheet structure of individual barnase with unique contribution of Trp94 [36]. The signal of negative ellipticity of VH barnase at 218 nm (–6000 deg·cm^2·dmol^{-1}) is significantly higher than that of barnase, which reflects unusually high, in comparison with other immunoglobulin domains, negative ellipticity of the VH domain (–8300 deg·cm^2·dmol^{-1}). The latter originates from distortion of secondary structure of the VH domain under conditions of partial loss of tertiary interactions that are essential for maintenance of β-sheet structures [10]. The theoretical circular dichroism spectrum calculated using spectra of the individual barnase and the VH domain differs from the experimental spectrum of the chimeric protein (Fig. 4b). This suggests that fusion of the VH domain and barnase in the chimeric protein resulted in changes in its secondary structure. However, these changes are less pronounced than the changes in tertiary structure of VH–barnase detected by means of calorimetry.

In the near ultraviolet region, the CD spectrum of VH–barnase has a global minimum at 265 nm and additional minimum at 275 nm (Fig. 5). This indicates the presence of compact tertiary structure and is quite consistent with the results obtained by scanning calorimetry and fluorescence. Chemical denaturation flattens the spectrum, and this suggests loss of asymmetric environment of aromatic amino acids. Thus, the spectroscopic results

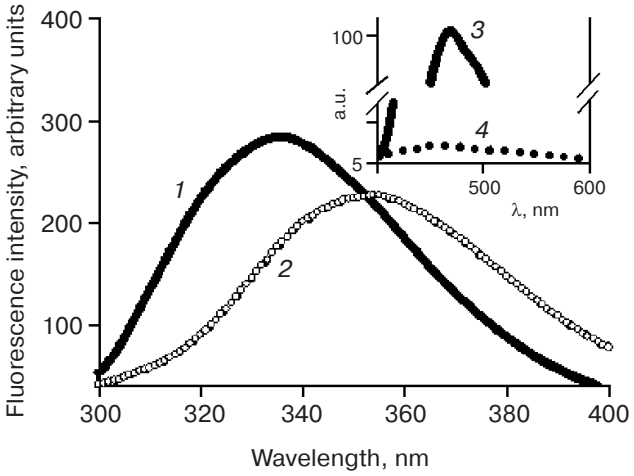

Fig. 3. Fluorescence spectra of VH–barnase in 0.05 M sodium phosphate buffer, pH 7.4 (*1*), and in 6 M GdnHCl (*2*). Inset: fluorescence spectra of ANS in the presence of VH–barnase in 0.05 M sodium phosphate buffer, pH 7.4 (*3*), and in 6 M GndHCl (*4*).

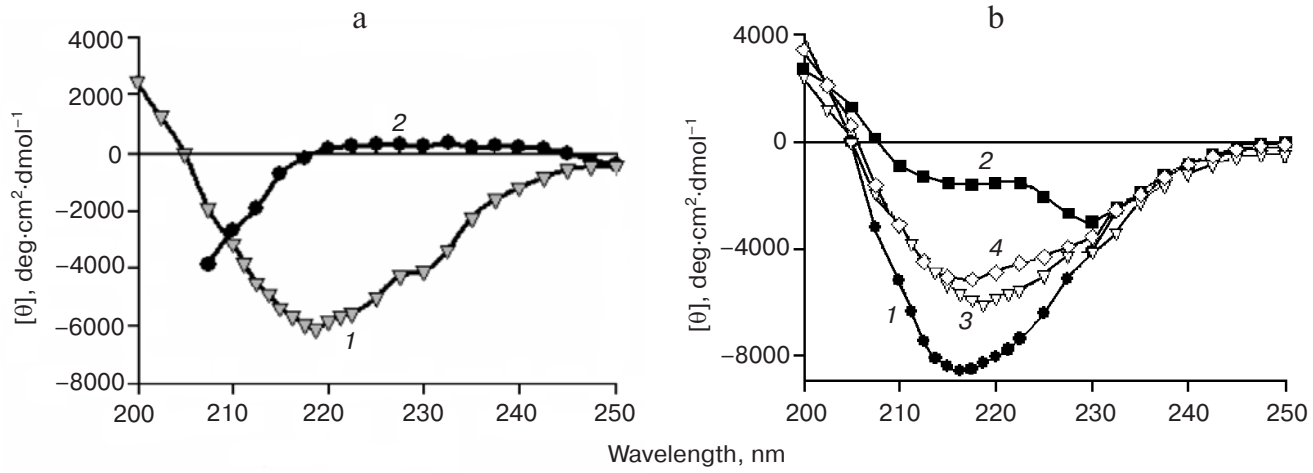

Fig. 4. CD spectra in the far ultraviolet region: a) VH barnase in 0.05 M sodium phosphate buffer, pH 7.4 (*1*), and in the presence of 6 M GdnHCl (*2*); b) VH domain (*1*), barnase (*2*), and VH–barnase (*3*), theoretical spectrum VH domain + barnase (*4*) calculated on the basis of CD spectra of the individual proteins.

indicate that VH–barnase has well-defined secondary structure, together with compact but partially unfolded tertiary structure.

Functional activity of VH–barnase. RNase activity of VH–barnase (expressed as grams of degraded RNA per second) per mole of barnase subunit was 9.3 $g \cdot sec^{-1} \cdot mol^{-1}$; this is two times less than the activity of isolated barnase (21.6 $g \cdot sec^{-1} \cdot mol^{-1}$) [13]. This suggests altered functional activity of barnase in the chimeric protein.

The antigen binding activity of VH–barnase was determined by ELISA. The chimeric protein binds human ferritin with binding constant $K_a = (1.0 \pm 0.2) \cdot 10^7$ M^{-1} (Fig. 6), which is four time less than the K_a value obtained for the individual VH domain [10]. This

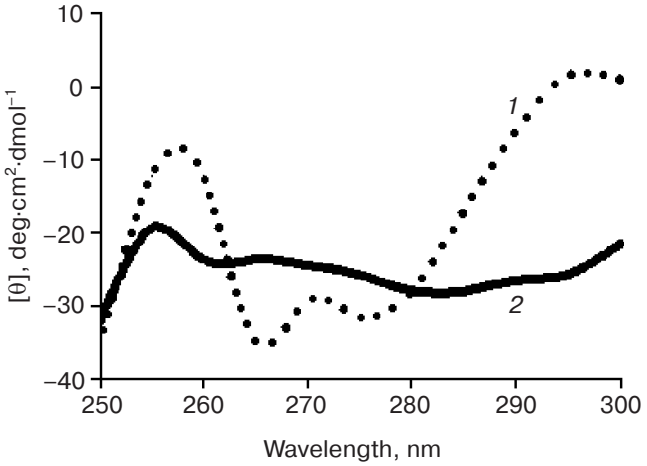

Fig. 5. CD spectra in the near ultraviolet region: barnase in 0.05 M sodium phosphate buffer, pH 7.4 (*1*), and in the presence of 6 M GdnHCl (*2*).

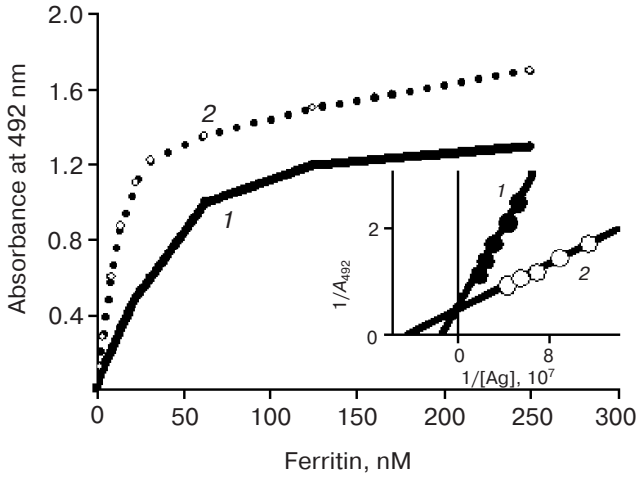

Fig. 6. Antigen binding activity of VH–barnase (*1*) and VH domain (*2*). VH–barnase or the VH domain was adsorbed onto the surface of polystyrene plate wells, and then increasing amounts of ferritin were added. Bound ferritin was determined by means of the antiferritin antibody G10 conjugated with horseradish peroxidase. The K_a values were calculated from double reciprocal plots (inset).

provides further evidence for changes in the antigen binding site of the VH domain of the chimeric protein. Thus, both the VH domain and barnase partially loose their activity when fused into the chimeric protein.

DISCUSSION

Using scanning calorimetry and fluorescence and CD spectroscopy, we have demonstrated here that fusion of barnase with the VH domain in the chimeric protein

resulted in changes in properties of both domains and induced formation of partially unfolded but functionally active conformation. The chimeric protein forms a single calorimetrically detectable domain (so-called calorimetric domain) with conformation that corresponds to a molten globule. The conformation of VH–barnase is characterized by:

– well-defined secondary structure;

– compact but partially unfolded tertiary conformation with partial exposure of hydrophobic clusters under physiological conditions and by low thermodynamic stability with an extremely low calorimetric enthalpy of denaturation.

In spite of partially unfolded conformation and fusion of the VH domain with barnase into the single calorimetric domain, VH–barnase preserves both antigen binding and RNase activities of the two constituent domains. However, loss of a significant part of structural interactions in each protein module of the fusion results in fourfold decrease in RNase activity and twofold decrease in antigen binding constant.

Our conformational study of VH–barnase provides one of the first experimental results obtained for the family of intrinsically disordered proteins. These results demonstrate that the members of this protein family can possess two different activities. Moreover, the results show that two activities of the chimeric protein can be preserved in partially unfolded tertiary structure distorted by potent nonspecific interactions of the two constituent domains.

Given the loss of a significant part of structural interactions of VH–barnase, one of the goals of this study was to determine whether structural organization of the chimeric protein can be increased by changes in refolding conditions *in vitro* that would result into formation of more compact conformation. To address this problem, we varied conditions of protein folding *in vitro* by means of various chemical additions known to increase folding efficiency and also by additions of GroES and GroEL chaperones.

We found that the amount of structure of VH–barnase is under thermodynamic rather than kinetic control and therefore it does not depend on folding conditions. However, kinetically controlled aggregation of partially unfolded species of the chimeric protein significantly alter the yield of its final conformation during folding. As a thermodynamic trap that prevents folding of VH–barnase into a more compact structure, one could suggest interaction of VH domain (existing as the molten globule) with "premature" partially unfolded structures of barnase. These partially unfolded structures might include so-called "nucleation sites" that maintain a native-like topology even under denaturing conditions and constitute folding nuclei [37].

When considering distinct results of protein engineering obtained for VL–barnase that demonstrates the chaperone-like effect of RNase in comparison with VH–barnase lacking this effect, one should take into account that the isolated VH domain of monoclonal antibody F11 is a functionally active molten globule lacking a significant part of stabilizing interactions and therefore representing a "poor" partner for folding. We suggest that the ability of barnase to increase solubility of antibody domains requires a certain minimum of productive structural interactions, and in the case of the VH domain this minimum has not been achieved.

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