Fusion of the antiferritin antibody VL domain to barnase results in enhanced solubility and altered pH stability

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Introduction
Antibody fragments fused to toxins constitute a novel class of chimeric molecules designed for a potential application in targeted cancer therapy. Immunotoxins that comprise variable antibody VH and VL domains fused to the toxic and translocation domains of Pseudomonas exotoxin (Chaudhary et al., 1989; Brinkmann, 1996) were recently applied, with a high therapeutic response rate, for treatment of patients with hairy cell leukemia (Kreitman et al., 2001). Another class of immunotoxins employs plant toxins (e.g. ricin, viscinum, saporin, gelonin) that generally belong to a subgroup of ribosome-inactivating proteins, as reviewed by Thrush et al. (1996) and Carter (2001). Furthermore, it was shown that RNases can be used to design immunotoxins in place of bacterial or plant toxins. Saxena et al. (1991) constructed a chimeric toxin composed of Pseudomonas exotoxin A and barnase, bacterial RNase from Bacillus amyloliquefaciens, and demonstrated cytotoxicity of bovine pancreatic RNase A after micro-injection directly into Xenopus oocytes. Prior et al. (1991) constructed a chimeric toxin of Pseudomonas exotoxin A and barnase, bacterial RNase from Bacillus amyloliquefaciens, and demonstrated cytotoxicity for murine fibroblasts. More recently, several members of the RNase family, including fungal, mammalian and human enzymes, were used for construction of cytotoxic anti-tumor immunoconjugates (Bettet al., 1992; Lacetti et al., 1992; Rybak et al., 1992; Deonarain and Epenetos, 1995, 1998; Jinno et al., 1996; Ratore and Batra, 1996; Deonarain et al., 1997; Zewe et al., 1997; Dubel, 1999; Gho and Chae, 1999; Suzuki et al., 1999; Yoon et al., 1999; Goyal and Batra, 2000; Psarras et al., 2000; Huhn et al., 2001; De Lorenzo et al., 2002a,b). Finally, RNase-based immunotoxins were reported to enter preclinical studies (Newton et al., 2001a,b), and RNases are now considered as a promising class of cytotoxic modules to design immunotoxins (Makarov and Ilinskaya, 2003; Newton et al., 2003). Human and mammalian RNases are expected to provide low immunogenicity (Rybak et al., 1992; Deonarain and Epenetos, 1994, 1998; Hursey et al., 2002) while barnase is thought to be more easily expressed in bacterial expression systems and to escape from mammalian RNase inhibitors.

Barnase is a rather small (110 amino acids, ~12.4 kDa) protein which is extensively characterized, including X-ray crystallography (Martin et al., 1999 and references cited therein), thermodynamic stability (Martinez et al., 1994), NMR-resolved solution structure (Bycroft et al., 1991), and the role of subdomains in folding/stability (Sancho and Fersht, 1992; Kippen et al., 1994). A useful feature of barnase is the presence in bacteria of a potent and specific inhibitor, barstar. A remarkably high barnase–barstar association constant [10^{-14} M^{-1} (Hartley, 2001)] provides an advantage of inhibiting barnase toxicity during expression in bacterial host cells.

The most frequently used design of an immunotoxin recognition module involves scFv antibody fragments composed of antibody variable VH and VL domains covalently

Chimeric immunotoxins that combine antigen recognition domains of antibodies and cytotoxic RNases have attracted much attention in recent years as potential targeted agents for cancer immunotherapy. In an attempt to obtain a structurally minimized immunofusion for folding/stability studies, we constructed the chimeric protein VL–barnase. The chimera comprises a small cytotoxic enzyme barnase, ribonuclease from Bacillus amyloliquefaciens, fused to the C-terminus of the light chain variable domain (VL) of the anti-human ferritin monoclonal antibody F11. While the individual VL domain was expressed in Escherichia coli as insoluble protein packed into inclusion bodies, its fusion to barnase resulted in a significant (~70%) fraction of soluble protein, with only a minor insoluble fraction (~30%) packed into inclusion bodies. The in vivo solubilizing effect of barnase was also observed in vitro and suggests a chaperone-like role that barnase exerted with regard to the N-terminal VL domain. Cytoplasmic VL–barnase was analyzed for structural and functional properties. The dimeric state of the chimeric protein was demonstrated by size-exclusion chromatography, thus indicating that fusion to barnase did not abrogate the intrinsic dimerization propensity of the VL domain. Ferritin-binding affinity and specificity in terms of constants of association with isoferritins were identical for the isolated VL domain and its barnase fusion, and RNase activity remained unchanged after the fusion. Intrinsic fluorescence spectra showed a fully compact tertiary structure of the fusion protein. However, significantly altered pH stability of the fusion protein versus individual VL and barnase was shown by the pH-induced changes in both intrinsic fluorescence and binding of ANS. Together, the results indicate that VL–barnase retained the antigen-binding affinity, specificity and RNase activity pertinent to the two individual constituents, and that their fusion into a single-chain chimeric protein resulted in an altered tertiary fold and pH stability.

Keywords: chaperone-like function/immunotoxin/protein stability/RNase/VL domain
linked through a flexible peptide (Brinkmann, 1996; Hudson, 1999). scFvs were considered minimal antibody fragments that retain both the affinity and specificity of parent molecules. However, high-affinity ‘single-domain antibodies’ comprising one single VH domain were recently described (Davies and Riechmann, 1996; Riechmann, 1996; Riechmann and Muyldermans, 1999; Wirtz and Steipe, 1999; Spinelli et al., 2000; Desnyter et al., 2001). Although VL domains were generally not considered potent antigen-binding units, a few examples of functional VL domains were reported. First, Gavish et al. (1977) showed functionality of the VL dimer derived by proteolysis from the dinitrophenyl-binding protein 315. More recently, Brinkmann et al. (1993) demonstrated that the immunotoxin comprising the VL domain and truncated Pseudomonas exotoxin displayed higher antigen-binding affinity than the VH-based immunotoxin. We showed that the individual VL domain of the anti-ferritin antibody F11 preserved its immunoglobulin fold (Martsev et al., 1998, 2000, 2002a) and antigen-binding function, with only ~2-fold decrease in affinity versus the individual VH domain (Martsev et al., 2002b) and ~7-fold decrease versus the scFv fragment (Martsev et al., 2000). Ferritin is a tumor-associated marker for human hepatocellular carcinoma (Hann et al., 1989), myeloid leukemia and Hodgkin’s disease (Aulbert and Schmidt, 1985; Moroz et al., 1987), and head and neck squamous cell carcinoma (Shikani et al., 1992). Radiolabeled full-length antibodies to human ferritin were successfully applied for immunotherapy of inoperable hepatocellular carcinoma and Hodgkin’s disease (Order, 1990; Hoefnagel, 1991; Lai et al., 1999; Vriesendorp et al., 1999).

In this work, we describe functional and structural features of a new immunofusion, VL–barnase, that comprises barnase fused to a single antibody VL domain derived from the anti-human ferritin antibody F11. Although the chimeric protein has potential applicability for tumor targeting, here we primarily consider it as a model of a structurally minimized immunotoxin suitable for extended folding/stability studies. As a first step towards this end, we demonstrate that fusion to barnase increased the solubility of the VL domain both in vivo and in vitro, and that the fusion protein retained the two functional activities as well as dimerization propensity of individual VL. Furthermore, we describe fusion-induced changes in pH stability of the chimeric protein.

Materials and methods

Construction of VL–barnase expressing plasmid

The VL domain–barnase fusion encoding plasmid pET(VL–barnase) (Figure 1) was generated by the standard techniques from the pETscF11 plasmid (Martsev et al., 1998) that encodes the antiferritin scFv fragment F11 comprising the VH domain followed by the linker peptide and the VL domain, and pMT413 plasmid (Hartley, 1988) that encodes the barnase–barstar cassette. Briefly, the VL-encoding fragment was amplified by PCR from pETscF11 DNA using a direct primer (5′-CTGGAATTCGCCCTGCTACAGATCAGCAGACA-3′) comprising the Ndel restriction site and a reverse primer (5′-ATGTTAGATTTCTTCTAAGTTGCAAGGCGCTCGACGGTGACAGG-3′) comprising the EcoRI restriction site. The amplified V1 gene was digested with Ndel and EcoRI and ligated into the Ndel/EcoRI-digested plasmid pET22b+ under the control of T7 RNA polymerase promoter. The structure of the resulting plasmid pET22VL and the absence of any mutations in the VL gene were confirmed by sequencing. The barnase gene was amplified by PCR from pMT413 DNA using a direct primer (5′-CTGGAATTCGCCCTGCTACAGATCAGCAGACA-3′) comprising the EcoRI restriction site and a reverse primer (5′-GTTTTAAGCCTAGGGATCCGGACGCTGCGTGA-3′) comprising the HindIII restriction site. The PCR product and the plasmid pET22VL were digested with EcoRI/HindIII and ligated. The ligation mixture was used to transform Escherichia coli TOP10 cells. Sequencing of six randomly chosen clones revealed that they all bore the expected mutations in the barnase encoding segment. The intermediate plasmid pBaH1-1 derived from the pMT413 plasmid (Hartley, 1988) was constructed to add a six-histidine tag to the C-terminus of barnase. The plasmid pET(VL–barnase) encoding the VL domain fused to intact barnase was obtained by ligation of Smal/HindIII digestions of DNA of one mutated clone and the Smal/HindIII fragment of the plasmid pH1-1. The resulting plasmid, in addition to the VL–barnase encoding insert, comprised the barstar gene under the control of its own promoter. The correct design and absence of any mutations in the barnase-encoding segment of the pET(VL–barnase) DNA were confirmed by sequencing.

Expression of the fusion protein

Escherichia coli BL21(DE3) cells transformed with the pET(VL–barnase) plasmid were grown in LB medium containing 100 µg/ml ampicillin at 37°C until the culture reached mid-log phase. After 3–4 h of growth, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and the cells were grown for an additional 3–5 h at 37°C. The bacterial cells were harvested by centrifugation at 3000 g for 15 min, resuspended in 0.1 M Tris–HCl, pH 8.1, and disintegrated by sonication. Inclusion bodies were separated from the soluble fraction of the cell lysate by centrifugation for 30 min at 40 000 g. Typically, the overall yield of VL–barnase expressed both in cytoplasm and inclusion bodies was ~40 mg per liter of the cell culture.

Barnase was obtained from TG1 cells as described by Hartley and Rogerson (1972).
Purification and refolding of VL–barnase

The soluble protein fraction was dialyzed against 7 M urea in 25 mM Tris–HCl, pH 7.0 (binding buffer), and applied on the Ni-NTA Sepharose column equilibrated with the same buffer. After washing the column with 10 volumes of the binding buffer, five volumes of the same buffer containing 3 M GdnHCl were passed over the column to remove barstar that was involved in a complex with VL–barnase and did not comprise a poly-His tag. Purified VL–barnase was eluted with 0.1 M imidazole in the binding buffer. The eluted protein did not contain a barstar contamination, as judged by SDS–PAGE, and was refolded by dialyzing against 0.1 M sodium phosphate for 16 h. A typical final yield of the refolded protein was ~15 mg from 1 l of the cell culture with a purity of not less than 95% as determined by SDS–PAGE.

Rapid in vitro refolding

VL–barnase and the individual VL domain in varying concentrations were initially dialyzed for 16 h against 0.1 M sodium phosphate, pH 7.4, containing 6 M urea. Refolding was performed by rapid one-step dialysis against 0.1 M sodium phosphate, pH 7.4. After centrifugation at 40 000 g for 30 min, the protein concentration in the supernatant was determined by measuring the UV absorbance at 280 nm.

Molecular mass determination

Gel filtration of VL–barnase was carried out on a Sephacryl S200 column (1.0×106 cm) equilibrated with 0.1 M sodium phosphate buffer, pH 7.4. The following molecular mass markers were employed: IgG (150 kDa), BSA (66 kDa), ovalbumin (45 kDa), lysozyme (14 kDa). Samples of 0.5 ml containing from 0.25 to 1.0 mg of VL–barnase, barnase or the VL domain were loaded on the column. All experiments were carried out at a room temperature with a flow rate of 10 ml/h.

Binding affinity and specificity

The antigen-binding affinity and specificity were determined in an enzyme-linked immunosorbent assay (ELISA) using the polystyrene-adsorbed VL domain or VL–barnase and varying amounts of human ferritins, spleen ferritin comprising ~85% L-subunit and ~15% H-subunit, and recombinant L-ferritin (~15 mg from 1 l of the cell culture with a purity of not less than 95% as determined by SDS–PAGE).

for 5 min. The reaction was stopped by adding 0.05 ml of 10% H2SO4, and the absorbance at 492 nm was determined.

Determination of ribonuclease activity

Quantitative RNA-degrading assay for VL–barnase and individual barnase was performed at 25°C according to Kunitz (1946) by spectrophotometrically measuring the decrease in RNA absorbance at 300 nm in a 1 cm pathlength cuvette containing 1 mg/ml yeast RNA dissolved in 0.1 M sodium phosphate, pH 7.4.

Fluorescence measurements

Intrinsic fluorescence spectra of the proteins were recorded in a 1 cm thickness quartz cuvette on an SFL-1211 fluorometer (Solar, Minsk, Belarus) at a protein concentration of 0.05 mg/ml in 0.1 M citrate–phosphate buffer (pH 2.0–6.0), 0.1 M sodium phosphate buffer (pH 6.2–7.8) and 0.1 M Tris buffer (pH 8.0–10.0). Fluorescence was excited at 280 nm. Spectra were corrected for the buffer blank.

Fluorescence of protein-bound ANS was recorded from 400 to 600 nm after excitation at 360 nm, as we previously described (Martsvev et al., 1998). The protein concentration was 0.05 mg/ml in the above buffers, and the molar ANS-to-protein ratio was 10:1 for individual barnase and the VL domain, and 20:1 for VL–barnase. For VL and VL–barnase, a monomeric molecular mass was taken for these calculations. Spectra were corrected for the ANS fluorescence without a protein.

Other methods

Expression and purification of the VL domain was performed as described in Martsvev et al. (2000). Methods for preparation of human spleen ferritin (Martsvev et al., 1995) and recombinant human L-ferritin (Santambrogio et al., 1993) were previously reported. SDS–PAGE was performed according to Laemmli (1970), and gels were stained with Coomassie Brilliant Blue R-250. The molecular weight of the fusion protein calculated from the amino acid sequence was 26 390 Da. The concentration of VL–barnase was determined from the UV absorbance at 280 nm using an extinction coefficient of 1.55 mg⁻¹ ml⁻¹ cm⁻¹; the latter was calculated according to Gill and von Hippel (1989) based on the known amino acid composition. Disulfide groups and free thiols were determined by the methods of Thanhauser et al. (1984) and Ellman (1959), respectively.

Results

Inhibitory expression system

Expression of functional barnase is lethal to the host cell but can be suppressed by co-expression of the barnase inhibitor, barstar, using the inhibitory expression system (Prior et al., 1996; Deyev et al., 1998; Hartley, 2001). We employed the expression system (Figure 1) that allowed constitutive transcription of the barstar gene under the control of its own promoter in order to provide basal synthesis of the inhibitor. Barstar was expected to readily form the barstar–barnase inhibitory complex, thereby protecting the host cell RNA from degradation by barnase and allowing expression of soluble VL–barnase. We obtained sufficient amounts of cytoplasmic VL–barnase (Figure 2), which itself provides indirect evidence for the formation of the barnase–barstar complex. As further evidence for the inhibitory complex formation, we observed that complete removal, by the Ni-NTA chromatography, of barstar that lacks the polyhistidine tag required strong denaturing conditions (7 M urea with 3 M GdnCl, Figure 3). When the soluble cellular extracts were chromatographed...
either in the absence of denaturants or in 7 M urea alone, the SDS–PAGE analysis of the 0.1 M imidazole eluate revealed, in addition to VL–barnase band, the presence of the prominent barstar band with an $M_r$ of ~10 kDa (data not shown).

Furthermore, when analyzing a number of transformants with a variable level of VL–barnase expression we did not observe a direct correlation between the amount of electrophoretically revealed barstar and VL–barnase. This latter finding suggests that barstar was normally expressed in excess of VL–barnase and protected the host cell from barnase toxicity.

**Structural composition of VL–barnase**

The sequence of the VL domain fused to barnase differed from that of individual VL in a single feature. In the individual VL domain, the C-terminal hexahistidine tag is present while in VL–barnase this tag was transferred to the C-terminal position of barnase (Figure 1). The peptide employed to link the C-terminus of VL with the N-terminus of barnase comprised 13 amino acids, Asp–Leu–Gly–Thr–Val–Asp–Ser–Ala–Ser–Ala–Gly–Ile–Leu. This hydrophilic non-repeating linker was used to provide enough flexibility of the fusion partners.

Determination of the number of disulfides under denaturing conditions according to Thannhauser et al. (1984) gave a value of $0.97 \pm 0.05$ mol per mol of VL–barnase. This result is consistent with a single disulfide present in the VL moiety of VL–barnase, with no Cys residues present in barnase. Ellman's method (Ellman, 1959) revealed no free thiols in the fusion protein. Our determinations of disulfides and free thiols for the individual VL domain gave the same results (Martsev et al., 2000). The data suggest the correct fold of the VL moiety, with the two Cys residues specifically bridged into a single disulfide bond and no free thiols or stable oxygen derivatives present. Thus, specific disulfide bonding of VL is unaltered by fusion to barnase.

**Fusion to barnase increases solubility of the VL domain in vivo**

While the individual VL domain was expressed as an insoluble protein packed into inclusion bodies, fusion of VL to barnase resulted in appearance of a significant fraction (60–70%) of the soluble chimeric protein in the cytoplasm (Figure 2). Another fraction (30–40%) of totally expressed VL–barnase formed insoluble inclusion bodies and was not analyzed in this work. This finding indicates that the barnase module provided in vivo attaining and stabilizing of a soluble conformation of the fusion protein, a process that did not occur with the individually expressed VL domain. This property of barnase is similar to an in vivo chaperone-like effect recently described for the maltose-binding protein (Kapust and Waugh, 1999) and some other polypeptides that are referred to as solubilizing proteins (Bach et al., 2001). It is noteworthy that barnase does not
include disulfide bonds, and its folding cannot therefore be affected by the reducing conditions of the cytoplasm. In this regard, barnase meets the requirement for solubilizing proteins that are frequently fused to a passenger protein in order to increase the in vivo yield of the soluble form of the passenger under reducing conditions of the cytoplasm.

**High yield of in vitro refolding for VL–barnase versus isolated VL**

Given the chaperone-like effect of barnase in vivo, we further investigated the in vitro solubilizing effect of barnase in rapid refolding experiments that involved single-step dialysis with subsequent quantification of the soluble protein (Table I). At low protein concentrations (0.1–0.3 mg/ml) that generally prevent or greatly diminish aggregation, the refolding yield for VL–barnase was similar to that obtained for individual VL. However, at higher concentrations, from 0.8 to 1.2 mg/ml, we observed a significantly higher refolding yield for the fusion protein. The increase in the amount of the soluble protein was accompanied by a decreasing amount of insoluble aggregates (data not shown). The results indicate that barnase fused to the VL domain significantly increased the yield of the soluble protein in vitro through preventing aggregate formation.

**Dimeric state of VL–barnase**

SDS–PAGE of VL–barnase displays a polypeptide band with an $M_r$ of ~26 kDa, fully consistent with an $M_r$ calculated from the amino acid sequence (26.39 kDa). However, the gel permeation chromatography showed that VL–barnase is a dimeric protein with an apparent $M_r$ of ~53 kDa (Figure 4). We previously showed that the individual VL domain F11 forms a dimer under physiological conditions (Martsev et al., 2002a), consistent with the previous observations that many, yet not all, VL domains formed predominantly dimeric species (Maeda et al., 1976; Gavish et al., 1977; Zavyalov et al., 1977; Azuma et al., 1978; Raffens et al., 1998; Ionescu-Zanetti et al., 1999).

We did not find the dimeric species for individual ligand-free barnase in our gel permeation chromatography studies (Figure 4), although it was previously reported that barnase is capable of forming dimers when bound to its biological ligands (Buckley and Fersht, 1994) and even trimers during crystallization (Zegers et al., 1999). Together, our data suggest that the dimeric state of the chimeric protein resulted from the high intrinsic dimerization propensity of the VL domain that was not abrogated by fusing to barnase.

**Functionality of the VL–barnase fusion**

In enzyme immunoassay, VL–barnase is capable of binding to human spleen ferritin. Measuring the antigen-binding affinity (Figure 5) yielded virtually identical affinity constants for both the isolated VL domain and VL–barnase [(3.4 ± 0.6) × 10⁷ M⁻¹ and (3.3 ± 0.5) × 10⁷ M⁻¹, respectively]. These data suggest that the VL module in VL–barnase retained functional native or native-like conformation with an affinity identical to that of the isolated VL domain.

The binding specificity of VL–barnase was assessed through its ability to discriminate between structurally related but immunologically different human ferritins, spleen ferritin comprising ~85% L- and ~15% H-subunits, and recombinant L-ferritin (100% L-subunits). Previously, we have shown that the dimeric VL domain in isolation generates an antigen-binding site that is rearranged versus the native binding site generated by the VH–VL heterodimer (Nymalm et al., 2002). This rearranged binding site recognized human spleen ferritin but did not recognize recombinant human L-ferritin. Discrimination between L+H and purely L-ferritins constitutes a specific feature of the VL domain and was not observed for the parent full-length antibody F11 (Nymalm et al., 2002). In the current study, we observed a similar binding specificity for both individual VL and VL–barnase (Figure 5) because each of these two proteins displayed several-fold higher binding to spleen ferritin versus binding to recombinant L-ferritin. Together, these data strongly suggest that fusion to barnase did not alter the antigen-binding site of the VL domain.

The RNase activities of VL–barnase and isolated barnase were compared in terms of the rate of RNA degradation calculated per mole of the barnase monomer. The VL–barnase activity expressed in grams of RNA degraded in a second per mole of the barnase monomer was virtually identical to that of individual barnase (21.6 and 19.1 g s⁻¹ mol⁻¹, respectively), thus suggesting that functionality of the barnase module remained unaltered by fusion into the chimeric protein.

**Stability of tertiary structure**

The intrinsic fluorescence spectrum of VL–barnase at pH 7 suggests a compact tertiary structure with aromatic fluorophores protected from quenching by the solvent, as judged by the emission maximum at ~337 nm and a red shift to 355 nm after addition of 6 M GdnHCl (Figure 6A). On lowering the pH from 7 to 3, we observed a decrease in the fluorescence intensity at ~337 nm (Figure 6B) and a more pronounced red shift in the fluorescence maxima at 355 nm (Fig. 6C). The observed loss of fluorescence intensity is consistent with unfolding of the tertiary structure of VL–barnase (Zegers et al., 1999).

**Table I. Refolding yield of the individual VL domain and VL–barnase in vitro**

<table>
<thead>
<tr>
<th>Protein</th>
<th>$C_0$ (mg/ml)</th>
<th>$C_{in}$ (mg/ml)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VL</td>
<td>0.10</td>
<td>−0.10</td>
<td>&gt;95</td>
</tr>
<tr>
<td></td>
<td>0.30</td>
<td>0.28</td>
<td>93</td>
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<tr>
<td></td>
<td>0.50</td>
<td>0.31</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>0.80</td>
<td>0.33</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.39</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>1.3</td>
<td>0.40</td>
<td>30</td>
</tr>
<tr>
<td>VL–barnase</td>
<td>2.4</td>
<td>−2.4</td>
<td>&gt;95</td>
</tr>
</tbody>
</table>

$C_0$ and $C_{in}$ protein concentrations before and after refolding, respectively.
intensity that was attributable to enhanced accessibility of aromatic fluorophores to quenching by the solvent. The fact that neither the individual VL domain nor barnase significantly changed their fluorescence emission between pH 7 and 3 strongly suggests altered pH stability and therefore changes in a tertiary structure of the fusion protein versus the individual proteins, VL and barnase (Figure 6B). pH stability was further considered over the extended pH range by comparing the transition curves generated for VL–barnase and its individual constituents, VL and barnase (Figure 7A). The individual VL domain did not show any changes in fluorescence intensity between pH 3 and 10 while barnase demonstrated changes in emission spectra above pH 7. For VL–barnase, the midpoint transition was observed at pH 7.7 versus pH 8.3 observed for individual barnase. This difference in pH stability provides more evidence for fusion-induced conformational changes in the tertiary structure of VL–barnase.

The pH-induced unfolding of VL–barnase and the individual constituents, VL and barnase, was further analyzed by monitoring changes in fluorescence of ANS, the generally accepted probe for exposed hydrophobic surfaces on a protein. The hydrophobic dye ANS does not bind to proteins with native-like spatial structure, nor does it interact with unfolded polypeptides. None of the three proteins displayed binding to ANS at physiological pH (Figure 7B, inset) which is indicative of their native or native-like fold. However, on decreasing pH from 7 to 2, the three proteins demonstrated distinct changes in ANS binding (Figure 7B), which is most clearly seen in the two discriminatory regions, between pH 4 and 5 and in the pH 2–2.5 range. Between pH 4 and 5, ANS binding to the VL domain increased owing to the formation of the structural intermediate I1 with partially unfolded tertiary and native-like secondary structures (Martsev et al., 2002a). In this pH range, neither VL–barnase nor individual barnase bind ANS. In the second discriminatory region, binding of ANS to the VL domain decreased below pH 2.5 (Figure 7B), fully consistent with the formation of the less structured I2 intermediate state that possesses unfolded tertiary structure and distorted β-sheet...
secondary conformation (Martsev et al., 2002a). In contrast, binding of ANS to VL–barnase progressively increased between pH 2.5 and 2.0. These distinct changes in the ANS fluorescence could not be deduced from the behavior of the individual proteins, VL and barnase, given that barnase itself displayed high pH stability of the tertiary fold and therefore did not bind ANS over the pH 7–2 range (Figure 7B). This finding indicates that the T7 RNA polymerase reaction was not completely terminated at the end of the barnase gene, which resulted in a ‘bicistronic’ transcription of downstream located gene even though barstar was supplied with its own promoter. The inhibitory expression system yielded sufficient amounts (30–40 mg per liter of cell culture) of soluble cytoplasmic VL–barnase tightly associated with barstar. To disrupt the inhibitory complex and separate VL–barnase from barstar, we employed strong denaturing conditions similar to those previously applied by Prior et al. (1996) to separate barstar co-expressed with barnase fusions of the Pseudomonas exotoxin fragments. After removing the denaturant, the final yield of highly purified VL–barnase was ~15 mg per liter of cell culture.

**Discussion**

**Expression of VL–barnase**

*Escherichia coli* host cells could not tolerate a background level of barnase owing to the leaky expression pertinent to the T7 promoter. When we analyzed a number of clones transformed with the VL–barnase plasmid devoid of the barstar gene, the high expression level was observed only in the clones bearing functionally significant mutations in the barnase module. Therefore, barstar co-expression under the control of its own constitutive promoter was employed to suppress cytotoxicity of the barnase fusion. Interestingly, we observed an increase in the amount of barstar after induction with IPTG (Figure 2B). This finding indicates that the T7 RNA polymerase reaction was not completely terminated at the end of the barnase gene, which resulted in a ‘bicistronic’ transcription of downstream located gene even though barstar was supplied with its own promoter. The inhibitory expression system yielded sufficient amounts (30–40 mg per liter of cell culture) of soluble cytoplasmic VL–barnase tightly associated with barstar. To disrupt the inhibitory complex and separate VL–barnase from barstar, we employed strong denaturing conditions similar to those previously applied by Prior et al. (1996) to separate barstar co-expressed with barnase fusions of the Pseudomonas exotoxin fragments. After removing the denaturant, the final yield of highly purified VL–barnase was ~15 mg per liter of cell culture.

**Functionality of the two constituent modules**

The RNase activity studies and immunoassays revealed that both structural modules of the fusion protein preserved their biological functions. The RNase activity of VL–barnase was close to that of the individual enzyme. On the other hand, fusion of barnase to the VL domain did not result in any changes of the antigen-binding affinity. Moreover, fusion did not change the specificity of the VL domain, as demonstrated by the similar ability of the fused and individual VL domains to distinguish human isoferritins. Together, these data indicate the functional independence of the two heterologous modules fused into VL–barnase via the flexible linker peptide.

**Fusion-induced changes in stability of VL–barnase**

An unexpected finding was that pH stability of the fusion protein is markedly different from that of the two individual proteins. In this context, the dimeric state of the protein that yields the structure with the (VL–barnase). composition should not be disregarded, and the two possible types of interactions should be considered, namely, *trans* interactions between the identical barnase modules in the VL–barnase dimer, and *cis* interactions between VL and barnase. The presence within the VL–barnase dimer of barnase–barnase interactions that reduce the stability of the barnase module without altering the stability of VL was demonstrated by differential scanning calorimetry (Tsybovsky,Y.I., Kedrov,A.A. and Martsev,S.P., manuscript submitted for publication).

**Enhanced solubility of VL–barnase**

Fusion of the C-terminus of the VL domain to barnase resulted in at least two useful properties of the chimeric protein. First, the distribution of the expressed protein between the soluble cytoplasmic fraction and insoluble inclusion bodies was significantly shifted towards the cytoplasm whereas the individual VL domain was selectively accumulated in inclusion bodies. Secondly, barnase fused to the VL domain...
increased the yield of its in vitro refolding (Table I). This increase of solubility in vitro was dramatic upon refolding at relatively high concentrations, which is essential for large-scale preparation of barnase-based immunotoxins. The solubilizing effect of barnase observed both in vivo and in vitro suggests that barnase partially performs a chaperone-like function. A similar effect was previously described for fusions comprising maltose-binding protein, glutathione S-transferase and thioredoxin (Kapust and Waugh, 1999; Wang et al., 1999; Bach et al., 2001). It is noteworthy that all these solubilizing proteins were designed as N-terminal modules of the fusion constructs. It is generally believed that N-terminal modules are the first to fold upon protein synthesis on a ribosome and could therefore exert the chaperone-like effect through either shielding hydrophobic surfaces of, or providing additional charges to, a nascent polypeptide of the passenger protein. The former mechanism, however, implicitly presumes independent folding of the two modules, which cannot be a priori generalized. In contrast to the above examples, we describe here a similar chaperone-like effect with the construct bearing C-terminal barnase that would be the last module to fold, given the presumed independent (sequential) folding mechanism. The chaperone-like effect of C-terminal barnase suggests that this effect might involve a mechanism that does not strictly depend on the N- or C-terminal location of the constituent modules and, therefore, on the sequence of folding events. Alternatively, the chaperone-like effect might suggest that folding of the two heterologous modules in our fusion is not fully independent and involves interactions of the modules.

It is noteworthy that the homologous barnase–barnase interactions discussed above cannot per se explain the chaperone-like effect of barnase. Furthermore, unaltered RNase and antigen binding activities of the fusion protein suggest functional independence of the two heterologous modules within VL–barnase. Therefore, the chaperone-like effect might be attributed either to transient heterologous interactions that exist early in the refolding process but do not survive in the mature protein, or, alternatively, to additional charge and/or hydrophilicity conferred by fused barnase. In the context of the second suggestion, it is noteworthy that the individual VL domain in isolation comprises the hydrophobic amino acid-enriched surface that is hidden in the native full-length antibody by involvement with the VL–CL interface.

**Fusion to barnase does not alter the dimerization propensity of the VL domain**

VL–barnase formally follows a molecular design that can be considered minimal for immunotoxins because of the two lines of structural minimization. First, VL–barnase comprises only recognition and toxic modules, without a translocation domain employed in *Pseudomonas* exotoxin-based constructs (Batra et al., 1990; Brinkmann, 1996). Secondly, the recognition module is composed of a single variable domain of the antibody while other immunotoxins designed for in vivo applications generally comprise at least two recognition modules (Brinkmann, 1996; Hudson, 1999). However, the intrinsic propensity of the VL domain to dimerize resulted in a dimeric VL–barnase construct, which is in conflict with the aim of minimizing the molecular size of VL-based immunotoxins. One cannot rule out a possibility that the dimeric state of the VL domain is inherently related to retaining the antigen-binding function in the absence of the partner VH domain (Nymalm et al., 2002). In this context, minimizing the molecular size of VL-based immunotoxins would additionally require an attempt to destroy, by protein engineering, the dimerization propensity of VL without sacrificing the antigen-binding capacity. Interestingly, Ewert et al. (2003) reported that five out of seven VL domains considered in their work formed monomeric species at concentrations of ~5 μM. It is worth noting, however, that the molecular size is not considered a critical feature in recent attempts to enhance tumor targeting and/or internalization through designing bispecific/bivalent and multivalent antibody fragments that have from a two- to several-fold larger molecular size of a recognition module (Hudson, 1999; Nielsen and Marks, 2000; Willuda et al., 2001). Therefore, the high intrinsic dimerization propensity of VL domains does not interfere with their potential applicability as recognition modules of immunotoxins.

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**References**


