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Independent folding and conformational changes of the barnase module in the VL-barnase immunofusion: calorimetric evidence

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Abstract Although stability is critical for in vivo application of immunotoxins, a thermodynamic description of their folding/stability is still lacking. We applied differential scanning calorimetry (DSC) to RNase-based immunofusion comprising barnase, cytotoxic RNase from *Bacillus amyloliquefaciens*, fused to the light chain variable domain (VL) of anti-human ferritin antibody F11. By analyzing DSC curves recorded with or without preheating and addition of the barnase-stabilizing ligand guanosine 3′-monophosphate, we (i) assigned two well-resolved thermal transitions to the VL and barnase modules of VL-barnase, (ii) demonstrated independent folding of these two modules, and (iii) showed altered stability of the barnase module, which resulted from the dimeric state of VL-barnase.

Key words: Immunotoxin; RNase; Differential scanning calorimetry; Protein folding; Antiferritin

1. Introduction

Antibody fragments fused to toxins constitute a novel class of chimeric molecules designed for targeted cancer therapy. As an alternative to bacterial and plant toxins, RNases were shown to be promising toxic modules of immunotoxins, as reviewed in [1,2]. Several members of the RNase family including fungal, mammalian and human enzymes were fused to antibody fragments to obtain immunotoxins capable of killing tumor cells (e.g. see [3–9] and references therein). Bacterial RNase from *Bacillus amyloliquefaciens*, barnase, was used to provide cytotoxicity for murine fibroblasts [10]. Human and mammalian RNases are considered less immunogenic for humans [3,5,11,12], and barnase allows escape from RNase inhibitors present in mammalians [1,2]. Furthermore, barnase as a small (~12.4 kDa) and monomeric bacterial protein is thought to be more consistent with a bacterial expression system.

The most frequently used design of a recognition module in immunotoxins involves single-chain Fv (scFv) antibody fragments that comprise antibody heavy and light chain variable domains (VH and VL) linked through a flexible peptide into a single-chain protein ([13,14], for a review). However, Brinkmann et al. [15] demonstrated that the immunotoxin comprising the VL domain and truncated *Pseudomonas* exotoxin displayed higher antigen-binding affinity than the VH-based immunotoxin. We have recently shown that the recombinant VL domain derived from the anti-human ferritin antibody F11 [16,17] and fused to the N-terminus of barnase yielded a protein that retained both RNA-degrading and antigen-binding activities but possessed lower pH stability [18]. VL-barnase comprises a single-domain recognition module and a small cytotoxic domain, thus providing an immunotoxin design ideally suited for extended folding/stability studies.

Stability is a key feature that provides survival of immunotoxins following in vivo injection. An immunotoxin should possess enough stability under a variety of in vivo conditions that might result in aggregation at the physiological temperature, proteolytic degradation, and conformational alterations after internalization by a target cell. Furthermore, Willuda et al. [19] demonstrated that thermal stability of scFv fragments is critical for successfully targeting tumor cells. Moreover, completely folded conformation could not be generalized for all antibody variable domains, considering a large diversity of antigen-binding sites and their contribution to domain stability (e.g. see [16,17,20–22]). In this context, a high resolution method of differential scanning calorimetry (DSC) would provide a reliable means for description of folding and stability of chimeric immunotoxins and constituent domains. This method is of particular value for establishing the extent of structural interactions between constituent modules of chimeric immunotoxins and other multidomain proteins. In the only study known so far to directly address this matter in immunotoxins, Brinkmann et al. [23] demonstrated independent folding of the truncated *Pseudomonas* exotoxin and scFv fragment through functional measurements that revealed distinct folding kinetics for these two constituent modules. In the present work, we employed DSC for a description of folding and stability of VL-barnase fusion in comparison with the isolated individual components, the VL domain and barnase, with special emphasis placed on interactions between homologous and heterologous domains. We provide the first calorimetric demonstration for independent folding of the cytotoxic and antigen recognition modules in an RNase-based immunofusion. Furthermore, we demonstrate altered stability of the RNase module with no alterations in folding/stability observed for the recognition module.

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Abbreviations: GMP, guanosine 3′-monophosphate; scFv, single-chain Fv fragment; VH, antibody heavy chain variable domain; VL, antibody light chain variable domain

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2. Materials and methods

2.1. Expression and purification procedures

VL-barnase was expressed in *Escherichia coli* BL21(DE3) cells. In order to escape from potential cytotoxicity of barnase for the host cells, the expression plasmid pET(VL-barnase) comprised, in addition to the VL-barnase-encoding DNA insert, the gene of barstar, a small protein (89 amino acids, ~10 kDa) that constitutes a cytoplasmic inhibitor of barnase in bacterial cells. In similar inhibitory expression systems, barstar was expressed in excess to the barnase and readily formed a barstar–barnase complex, thereby protecting the host cell RNA from degradation by barnase [24–26]. Host cells transformed with the pET(VL-barnase) plasmid were grown in LB medium containing 100 mg/l of ampicillin at 37°C for 3–4 h, then isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM. After an additional 3–5 h at 37°C and centrifugation at 3000×*g* for 15 min, harvested cells were 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 expression procedure yielded 25–30 mg of soluble VL-barnase per liter of the cell culture. The soluble fraction comprising VL-barnase and co-expressed barstar was dialyzed against the binding buffer comprising 7 M urea in 25 mM Tris–HCl, pH 7.0, 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 guanidine hydrochloride were passed over the column to remove barstar that was involved in an inhibitory complex with VL-barnase and did not comprise its own poly-His tag. Purified barstar-free VL-barnase was obtained by elution of the column with 0.1 M of imidazole in the binding buffer. The eluate did not contain barstar contamination, as demonstrated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). VL-barnase was refolded by dialysis 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 not less than 95% as determined by SDS–PAGE.

Purification and properties of the recombinant VL domain F11 were previously described [16,17]. The VL domain was derived from the anti-human ferritin antibody F11 of mouse IgG2a/k subclass [27,28]. Barnase was obtained from TG1 cells as described by Hartley and Rogerson [29].

2.2. DSC

Measurements were performed with a DASM-4 scanning calorimeter (Biopribor, Pushchino, Russia) equipped with a computer interface in a temperature range of 10–80°C at a scan rate of 60 K/h. The reference cell was filled with the buffer used to dialyze the protein sample. The buffers used for the measurements were 0.1 M sodium phosphate in the pH interval 7.4–5.0, 0.05 M sodium phosphate adjusted with 0.02 M sodium citrate in the pH range 4.9–3.5, and 0.05 M NaH_2PO_4–HCl in the pH interval 3.4–2.0. The heat capacity curves were corrected for the instrumental baseline that was determined with both cells filled with the buffer. The protein concentrations varied between 0.5 and 1.8 mg/ml. The heat capacity curves were analyzed and deconvoluted using the algorithm of Privalov and Potekhin [30] and TERMCALC software supplied by the DASM-4 manufacturer. Variations of individual measurements for the midpoint transition temperature, T_m, were within 0.4°C.

2.3. Other methods

Protein concentrations were determined from the UV absorbance spectra using extinction coefficients calculated from the amino acid composition according to Gill and Von Hippel [31]. The extinction coefficients at 278 nm were 1.60 mg^{-1} ml cm^{-1} for VL-barnase, 2.15 mg^{-1} ml cm^{-1} for barnase and 1.22 mg^{-1} ml cm^{-1} for the VL domain.

3. Results

At pH 7.4, VL-barnase demonstrated a bimodal heat capacity curve with two well-resolved peaks centered around 47 and 60°C (Fig. 1). We did not observe changes of thermal transitions when the protein concentration varied in the range from 0.5 to 1.8 mg/ml. At neutral pH, repeated calorimetric recordings revealed poor reversibility for the high temperature transition and no reversibility at all for the low temperature one. Reversibility gradually increased as the pH decreased below 5. Reversibility is generally considered to be an essential [32], yet not critical [33–35], requirement for correctly measuring a transition enthalpy. At low pHs where thermal unfolding was reversible (Fig. 2A), the calorimetric scan yielded a single asymmetric peak that was resolved, by a deconvolution procedure, into two largely overlapping two-state transitions (Fig. 2B). Irreversibility of thermal transitions and their clear-cut resolution at neutral pH provide the two advantages for attaining the major aims of the study that involved (i) assignment of transitions to the two structural modules of VL-barnase and (ii) establishing the extent of their structural independence. As a third aim of the study, we assessed thermal stability and the amount of structure in VL-barnase versus the individual components, VL and barnase, through measuring T_m and partial heat capacity, C_p, parameters that do not critically depend on reversibility.

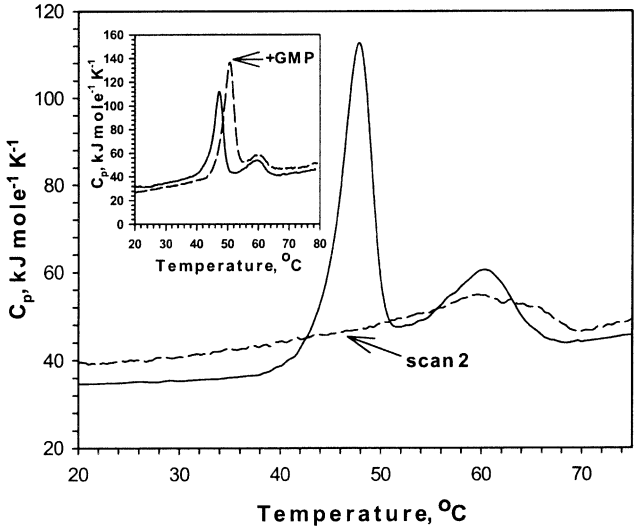

Fig. 1. Partial reversibility of thermal unfolding for VL-barnase at pH 7.4 (main panel) and selective stabilization of the barnase module (inset) by adding GMP.

The value of specific partial heat capacity of VL-barnase determined at 25°C, $C_p(25°C) = 1.34 \pm 0.13$ J/g/K, is typical for completely folded globular proteins [30,32]. This is indicative of the compact native-like fold of VL-barnase with no solvent-exposed hydrophobic amino acids, fully consistent with the lack of 8-anilino-1-naphthalene sulfonic acid binding shown for VL-barnase under physiological conditions [18].

Thermal unfolding of the isolated VL domain of the anti-ferritin antibody F11 occurred with a T_m around 60°C [16], consistent with what was reported for the human VL domain proteolytically derived from the Bence Jones protein IVA [36]. For VL-barnase at neutral pH, the second peak around 60°C is therefore attributable to the melting of the VL module. To unambiguously assign the first peak of thermal unfolding observed around 47°C, we analyzed thermal unfolding of VL-barnase in the presence of guanosine 3′-monophosphate (GMP), the barnase inhibitor that is known to tightly bind to barnase with enhancement of its thermal stability [37]. Adding GMP shifted the first peak up to 50°C (Fig. 1, inset), thus assigning this peak to thermal unfolding of the barnase

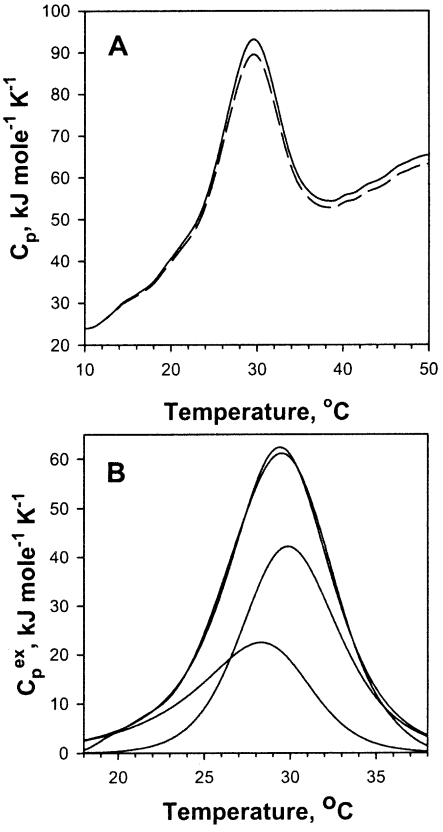

Fig. 2. Reversibility of thermal unfolding of VL-barnase at pH 2.0 as demonstrated by the two consecutive scans (A) and deconvolution of the excess heat capacity curve (B). The latter curve was derived from the heat capacity recording shown in A. The overall denaturation enthalpy (A) is 9.6 J/g. The enthalpies of the individual two-state transitions obtained by deconvolution (B) are 3.8 J/g and 5.8 J/g, and the T_m values are 28.2°C and 29.7°C.

module. Binding of GMP did not alter the second peak, consistent with its attribution to the melting of the VL module.

The significantly higher square under the first peak (Fig. 1) assigned to thermal unfolding of the barnase module is quite consistent with the unusually high calorimetric enthalpy of individual barnase. Previous calorimetric measurements at pH 5.0–5.5 [37,38] gave enthalpy values of 42.6–43.9 J/g, which are virtually identical to our data obtained with individual barnase under the same conditions (not shown). These values of unfolding enthalpy significantly exceed the range of 20–30 J/g generally found for calorimetric enthalpies of many globular proteins [30,32]. On the other hand, the thermal unfolding enthalpy for our VL in isolation (15.9 J/g at pH 5–7 [17]) is slightly below the lower limit of this range, which is again fully consistent with the smaller second peak (Fig. 1). Thus, distinct squares under the peaks provide further yet indirect support for their assignment.

The excess heat capacity curve of VL-barnase strongly differed from that of the equimolar mixture of barnase and the VL domain, as well as from the theoretical curve calculated using the two curves recorded for the individual components (Fig. 3A,B). In view of the closely similar calorimetric curves obtained for individual VL and the VL module involved into the fusion protein (Fig. 3B), this difference suggests conformational changes in the barnase module within the fusion versus individual barnase. One conformational feature that obviously contributes to these changes is a significantly lower thermal stability of the barnase module, as demonstrated by a decrease in the T_m (47°C versus 53°C observed for individual barnase). This thermal destabilization of the barnase module within VL-barnase after the fusion is an essential observation which is consistent with a fusion-induced decrease in pH stability of VL-barnase [18].

Interestingly, the T_m value of the barnase module of VL-barnase demonstrated an $\sim$5°C increase up to $T_m = 52$°C on going from pH 7.4 to pH 5.0, a property that we also observed for individual barnase (not shown). This increase in stability might be essential for in vivo application of barnase immunofusions considering that pH 4.6–5.5 or even lower was found in endosomes following protein internalization [39,40].

Is folding of the two heterologous modules of VL-barnase independent, or, alternatively, do folding-related interactions exist between the modules? To address this question, we employed irreversibility, at neutral pH, of the low temperature transition that belongs to unfolding of the barnase module (Fig. 4). After preheating of VL-barnase in a calorimetric cell up to 51°C followed by cooling down and a second heating, irreversibility resulted in disappearance of a calorimetrically revealed structure of the barnase module, with no changes observed for thermal unfolding of the VL module (Fig. 4B). These data strongly suggest independent folding of the two heterologous modules, VL and barnase, within

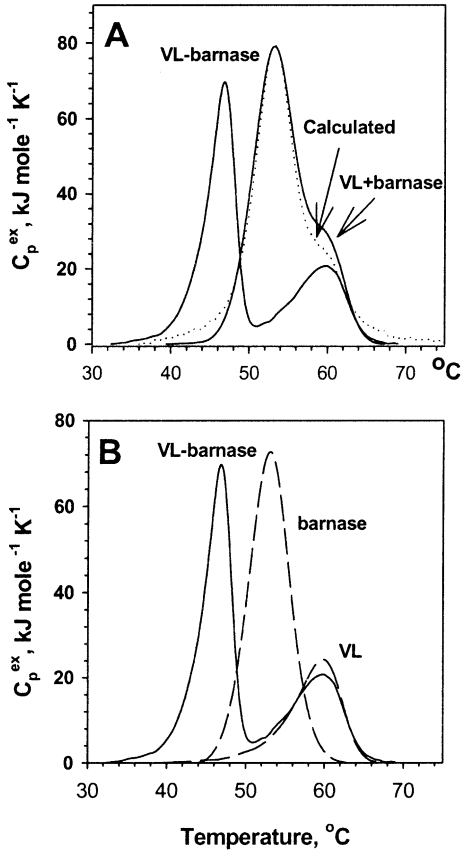

Fig. 3. A: Excess heat capacity curves obtained at pH 7.4 for VL-barnase, equimolar mixture of the VL domain and barnase, and the calculated curve generated from the sum of individual VL and barnase DSC curves. B: Excess heat capacity curves of VL-barnase (the same as in A) versus the individual components, VL and barnase, at pH 7.4.

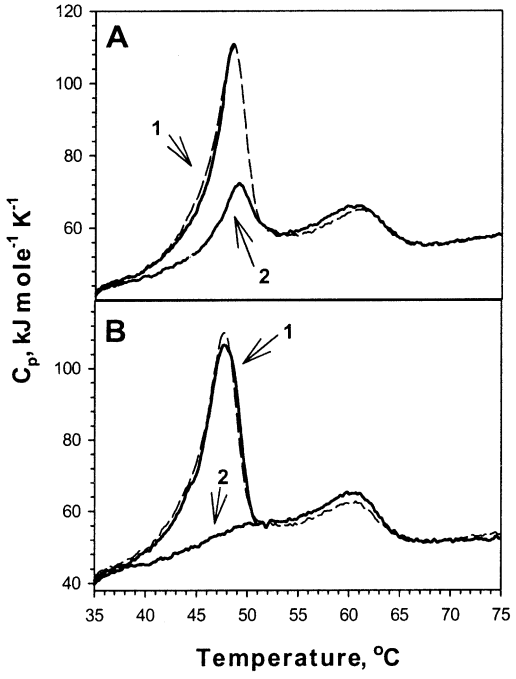

Fig. 4. Independent thermal unfolding of the barnase module in VL-barnase at pH 7.4. Solid lines: preheating (1) followed by cooling down and reheating over the full temperature scale (2). Dashed lines: thermal unfolding curves obtained without preheating. Preheating was stopped at either 48°C (A) or 51°C (B).

the fusion protein, consistent with their functional independence [18] and GMP-induced thermal stabilization of the barnase module that occurred independently of the VL module (Fig. 1).

4. Discussion

VL-barnase is a chimeric protein that belongs to a recently reported family of RNase-based immunofusions and involves the two heterologous proteins, a cytotoxic enzyme, bacterial RNase, fused to the VL domain derived from the anti-human ferritin antibody F11. Functional activities of the two heterologous modules of the chimeric protein remained unchanged versus individual VL and barnase [18]. Consistent with the functional independence of the two modules of VL-barnase, here we demonstrate their independent folding into a fully compact conformation. Retention of the two activities, antigen binding and RNA degradation, together with complete folding of the two structural modules constitute the two key features that are obligatory to consider RNase-based immunofusion a promising agent for tumor cytotoxicity studies.

Despite the structurally minimal design of our immunofusion that comprises the two single-domain modules, the chimeric protein dimerizes [18] due to the inherently high dimerization propensity of the VL domain [17]. The dimeric state of VL-barnase raises the question of whether homologous interactions between the two identical modules occur and, if they do, whether they alter stability. While the individual VL domain F11 retains the dimeric state both in isolation and within the fusion protein, individual ligand-free barnase is a monomeric protein. In the present work, we demonstrate that involvement of barnase into the dimeric VL-barnase construct resulted in a decrease of thermal stability. This decrease is attributed to the barnase module of the fusion protein, not to the VL moiety whose stability remained unaltered by fusion. To explain the markedly lower thermal stability of the barnase module within the dimeric VL-barnase, the two following observations should be considered. First, the isolated VL homodimer and the VL module within VL-barnase display the same calorimetric parameters, T_m and the square under the peak (Fig. 3B). Second, independent folding of the two heterologous modules, VL and barnase, revealed by both the repetitive scans of VL-barnase (Fig. 4) and GMP-induced changes in stability (Fig. 1), strongly suggests the lack of interactions between the two heterologous modules. Therefore, we have no alternative but to assign the thermal destabilization of the barnase moiety to homologous barnase–barnase interactions within the VL-barnase dimer. Destabilizing barnase–barnase interactions might either underlie or be related to the lower pH stability of VL-barnase that we have recently shown [18].

It is commonly believed that to provide enough stability after in vivo injection, a therapeutic protein should possess a T_m at least 10°C above the physiological temperature. In this context, a T_m of 47°C obtained for the barnase module of our VL-barnase implies marginal thermal stability, thus indicating that stabilizing mutagenesis of the barnase module would be beneficial. Interestingly, the barnase module appeared more stable (by $\sim$5°C in terms of T_m) at pH 5, the pH value which is close to that observed in endosomes following internalization of a ligand bound to its target on a cell membrane [39,40]. Therefore, the intracellular stability of VL-barnase might appear higher that the extracellular one.

In summary, using VL-barnase as a model of an immunotoxin suitable for folding/stability studies, we obtained the first calorimetric evidence for independent folding of cytotoxic and recognition modules of an immunofusion comprising RNase and an antibody fragment. Furthermore, we demonstrated that the VL-driven dimerization of the immunofusion destabilizes the RNase, not the VL moiety, through a mechanism that involves homologous interactions within the RNase module, with no interactions of the heterologous modules being observed.

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