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Optimization of the crystallizability of a single-chain antibody fragment

Single-chain variable antibody fragments (scFvs) are molecules with immense therapeutic and diagnostic potential. Knowledge of their three-dimensional structure is important for understanding their antigen-binding mode as well as for protein-engineering approaches such as antibody humanization. A major obstacle to the crystallization of single-chain variable antibody fragments is their relatively poor homogeneity caused by spontaneous oligomerization. A new approach to optimization of the crystallizability of single-chain variable antibody fragments is demonstrated using a representative single-chain variable fragment derived from the anti-CD3 antibody MEM-57. A Thermofluor-based assay was utilized to screen for optimal conditions for antibody-fragment stability and homogeneity. Such an optimization of the protein storage buffer led to a significantly improved ability of the scFv MEM-57 to yield crystals.

1. Introduction

Knowledge of protein three-dimensional structure is important for understanding protein function. The three-dimensional structure can, amongst other things, also provide a basis for protein-engineering approaches, especially in the case of antibody single-chain variable fragments (scFvs). X-ray crystallography is the principal method used in protein structure determination; close to 90% of all of the protein structures deposited in the Protein Data Bank to date have been determined using this method. The crystallization of the protein represents a common bottleneck in the process of structure determination, as a crystal is an essential requirement for diffraction experiments. The crystallization process is influenced by numerous factors, with the properties of the protein sample being the most important variable (Dale *et al.*, 2003). Therefore, pre-crystallization biochemical and biophysical characterization of the protein sample can help in successful crystallization (Zulauf & D'Arcy, 1992; Ericsson *et al.*, 2006). The use of pre-crystallization analysis is described here in detail for a single-chain variable fragment of the antibody MEM-57.

The monoclonal antibody MEM-57 (murine IgG2) recognizes the human CD3 antigen, which is expressed on the surface of T lymphocytes in complex with the T-cell receptor (Hilgert *et al.*, 1989). CD3 plays a role in the transduction of the activation signal after antigen recognition by the T-cell receptor (Weiss, 1990). The antibody MEM-57 shows similar effects (Hilgert *et al.*, 1989; Pan *et al.*, 2006) to the therapeutic anti-CD3 antibody OKT3 (Kung *et al.*, 1979; muromonab CD3, Orthoclone OKT3), which is used as an immunosuppressant in organ transplantation (Goldstein, 1987; Sgro, 1995; Chatenoud, 2003). The antibody MEM-57 could be used in diagnostics of autoimmune diseases, for T-cell lymphoma classification, as an immunosuppressant in transplantation (Hilgert *et al.*, 1989) or in cancer therapy in the therapeutic antibody format of a bispecific T-cell engager (Wolf *et al.*, 2005). Structural information on scFv MEM-57 would facilitate humanization of the antibody for therapeutic applications.

ScFv fragments are constructed as a fusion of the variable domains of the heavy (V_H) and light (V_L) immunoglobulin chains connected by a flexible linker, which stabilizes the noncovalent interaction between the V_H and V_L (Huston *et al.*, 1988; Bird *et al.*, 1988). The linker sequence (Gly₄Ser)₃ (Huston *et al.*, 1988) is universally used, as

Table 1

Sequence of the scFv MEM-57 recombinant fragment.

Heavy-chain variable domain (V _H)	EVQLQSSGGPEMPKPGASVKISCKATGYTFNRYIEWIKQR- PGHGLEWIGEILPGSGKTNYNKFKGKATFTADISAN- TVHTQISSLTSEDSAVYYCARAYRDGGYIMDYWGQG- TTVTVS
Linker	GGGSGGGSGGGSGGGSGGGSGG
Light-chain variable domain (V _L)	DIVMTQSPATLSVIPGDRVSLSCRASQSDRLSHWYQQKS- HESPRLLIKYASQSIGIPSRFSGSGSGSDFTLSINS- VEPEDGVVYCCQNGHSFPLTFGAGTKLEIKR
c-Myc epitope (cloning artifact)	EQKLISEEDL
His ₅ tag (cloning artifact)	NGT-HHHHH

it is flexible, hydrophilic, protease-resistant and does not interact with either domain (Papanastasiou *et al.*, 1999). A length of the linker of between 12 and 25 amino-acid residues (Jäger & Plückthun, 1999; Pantoliano *et al.*, 1991) allows the correct assembly of the two variable domains, resulting preferentially in the monomeric scFv form (Zdanov *et al.*, 1994; Whitlow *et al.*, 1994; Kortt *et al.*, 1994). However, a fraction of domain-swapped scFv dimers and higher oligomers can be also formed, which prevail when shorter linker sequences are used (Power *et al.*, 2003; Kortt *et al.*, 1997; Holliger *et al.*, 1993; Atwell *et al.*, 1999; Carmichael *et al.*, 2003). The preferred oligomeric form of the scFv depends not only on the length of the linker but, amongst other variables, also on the protein concentration and on the pH and the ionic strength of the storage buffer (Arndt *et al.*, 1998), which can be the subject of further optimization.

Here, we use the scFv MEM-57 fragment as a model to demonstrate the general approach of optimization of the storage-buffer composition for scFv fragments in order to enhance their crystallizability.

2. Experimental

2.1. Protein expression and purification

The coding sequences for IgG heavy-chain and light-chain variable domains were obtained from hybridoma total RNA using RT-PCR. The N-terminal primers corresponded either to the amino-acid sequence experimentally determined by N-terminal sequencing or, in the case of a blocked N-terminus, a mixture of degenerate primers (Orlandi *et al.*, 1989) was used. The C-terminal primers corresponded to the conserved sequence of the constant regions adjacent to the variable domains (Kabat *et al.*, 1991). The sequenced PCR products were re-amplified using modified primers to allow assembly of the (V_H)-(Gly₄Ser)₄-(V_L) scFv format, followed by a c-Myc tag sequence for anti-Myc antibody detection. Expression vectors were constructed using a modified pET-22b(+) vector, in which the scFv coding sequence is preceded by the pelB signal sequence, targeting the recombinant product into the periplasmic space, and is followed by a His₅ tag for purification.

The expression and isolation procedure was based on previously published protocols (Griep *et al.*, 1999; Kipriyanov *et al.*, 1997; Kaprálek *et al.*, 1991). *Escherichia coli* BL21 (DE3) cells transformed by an expression plasmid were inoculated to a final OD_{550 nm} of 0.1 in LB medium supplemented with 0.1 mg ml⁻¹ ampicillin and 0.4% (v/v) glycerol and were cultured at 310 K. After reaching an OD_{550 nm} of 1.0, the cell cultures were supplemented with IPTG to a final concentration of 0.25 mM to induce recombinant protein expression and were cultured at 293 K for an additional 4 h. The cells were harvested by centrifugation and the soluble recombinant protein was isolated from the periplasmic space by hypotonic shock. The cells were washed in cold hypertonic buffer [30% (w/v) sucrose, 50 mM Tris pH 8.0, 1 mM EDTA, 1 mM PMSF; 10 ml of buffer per gram of

cell paste] and centrifuged. The periplasmic space was then lysed by 45 min incubation at 273 K and 200 rev min⁻¹ in cold hypotonic buffer (5 mM MgSO₄, 10 mM Tris pH 8.0, 1 mM PMSF); a volume of 20 ml buffer was used per gram of cell paste. The periplasmic protein fraction was cleared by centrifugation, dialyzed against 50 mM NaH₂PO₄ pH 8.0, 300 mM NaCl at 277 K and loaded onto an Ni-CAM HC Resin column (Sigma-Aldrich, USA) at a flow rate of 1 ml min⁻¹. The His₅-tagged protein was eluted using a step gradient of imidazole (5, 20, 50 and 200 mM). The eluted protein was dialyzed against 20 mM diethanolamine pH 8.4 and further purified by ion-exchange chromatography using an ÄKTAbasic FPLC system on a 1 ml MonoQ 5/50 GL Tricorn column (Amersham Biosciences, UK) at a flow rate of 1 ml min⁻¹. The protein was eluted using a segmented gradient of NaCl: 0–200 mM NaCl in 20 ml, 200 mM–1 M NaCl in 10 ml, 1 M NaCl in 5 ml and 1–0 M NaCl in 5 ml.

The purified protein was concentrated by ultrafiltration using Amicon Ultra concentrators (Merck Millipore, USA) and stored in 20 mM diethanolamine pH 8.4, 100 mM NaCl or 100 mM sodium phosphate pH 7.5, 200 mM NaCl at 203 K. Protein purity was monitored by silver-stained SDS-PAGE under nonreducing conditions.

2.2. Size-exclusion chromatography

Analytical size-exclusion chromatography (SEC) was performed using an ÄKTAbasic FPLC system on a 23 ml Superdex 200 10/300 GL Tricorn column (Amersham Biosciences, UK) at a flow rate of 0.5 ml min⁻¹ in 20 mM diethanolamine pH 8.4, 100 mM NaCl or 100 mM sodium phosphate pH 7.5, 200 mM NaCl.

2.3. Thermofluor assay

The Thermofluor assay was performed according to a previously published protocol (Pisackova *et al.*, 2013; Procházková *et al.*, 2012) based on the procedure described previously (Ericsson *et al.*, 2006; Reinhard *et al.*, 2013). The stabilizing effect of 22 buffers at a concentration of 100 mM was tested in the presence or absence of 200 mM NaCl; the protein concentration was 0.2 mg ml⁻¹. The melting temperatures (*T*_m) were determined as the minima of the negative first derivatives of the melting curves using the *LightCycler* 480 Software (Roche, Switzerland).

2.4. Dynamic light scattering

Dynamic light-scattering (DLS) measurements were performed prior to setting up the crystallization trials using 15 µl concentrated (15 mg ml⁻¹) centrifuged protein samples at 532 nm, 90° angle and 293 K on a Laser-Spectroscatter 201 (GmbH Netzwerk RNA-Technologien, Germany).

2.5. Protein crystallization

The scFv MEM-57 protein at a concentration of 15 mg ml⁻¹ was used for initial crystallization screening. Screening was performed at 291 K by the sitting-drop vapour-diffusion method with the help of a Gryphon crystallization workstation (Art Robbins Instruments, USA) in 96-well sitting-drop Intelli-Plates (Art Robbins Instruments, USA) using the commercial screening kit The PEGs Suite (Qiagen, Netherlands). A total volume of 450 nl of a mixture of the protein and precipitant solution in a 2:1 ratio, respectively, was equilibrated against 50 µl reservoir solution.

Figure 1

Scheme of the scFv MEM-57 antibody fragment. (a) A scheme of the scFv MEM-57 gene construct and the corresponding protein product; V_H and V_L are the variable domains of the heavy and light immunoglobulin chains, respectively, pelB is a leader sequence targeting recombinant product to the periplasm, c-Myc is a ten-amino-acid epitope and His₅ is the pentahistidine purification tag. (b) Quaternary structure of the scFv MEM-57 protein. In addition to scFv monomers, domain-swapped inter-chain scFv dimers can be formed. (c) The purity of scFv MEM-57 is shown in lane 1 on a silver-stained denaturing SDS-PAGE gel under nonreducing conditions; the molecular-weight standard is in lane 3 (molecular weights are indicated in kDa) and lane 2 is empty.

3. Results and discussion

The single-chain variable fragment (scFv) of the antibody MEM-57 was constructed: variable domains of the heavy and light chains were joined by a flexible linker and further equipped with an N-terminal pelB leader sequence and a C-terminal c-Myc tag and His₅ tag. A longer version of the classical (Gly₄Ser)₃ linker (Huston *et al.*, 1988) with the sequence (Gly₄Ser)₄ was used to favour the formation of scFv monomers (Table 1, Fig. 1). Fig. 1(b) illustrates the formation of inter-chain domain-swapped dimers, a typical feature of scFv

fragments, which occurs in solution. To our knowledge, no other type of dimerization has been observed for scFv fragments.

The N-terminal pelB leader sequence targets the recombinant product into the periplasmic space of *E. coli* and is cleaved off during transport. In this compartment, disulfide bridges are formed and the recombinant antibody fragment usually accumulates in a soluble form. The recombinant protein was isolated through selective opening of the periplasmic compartment by osmotic shock. A two-step purification protocol employing nickel-chelation chromatography and ion-exchange chromatography produced a moderate yield of pure protein, as analyzed by nonreducing denaturing SDS-PAGE (Fig. 1c). The yield of 3 mg scFv MEM-57 per litre of bacterial culture is comparable to the typical yields obtained for other recombinant antibody fragments prepared from *E. coli* periplasm (Kipriyanov *et al.*, 1997; Bayly *et al.*, 2002). The antigen-binding activity of the scFv MEM-57 fragment was confirmed by flow cytometry on CD3-positive Jurkat cells (data not shown).

The ratio between the monomeric and the multimeric forms of the purified scFv fragment was determined by analytical size-exclusion chromatography (SEC) under native conditions. An equilibrium was established between monomer, dimer and higher oligomers of scFv MEM-57 in the original storage buffer, which was 20 mM diethanolamine pH 8.4, 100 mM NaCl (Fig. 2a). The monomeric form represented about 65% of the total protein. Dynamic light scattering (DLS) was also used to evaluate the dispersity of the protein preparation at the high concentration used for crystallization experiments. The DLS analysis revealed protein sample polydispersity (Fig. 2b), which indicated a low probability of successful crystallization of the protein (Zulauf & D'Arcy, 1992).

The equilibrium between oligomeric scFv forms was established even after the monomeric and dimeric forms were separated using ion-exchange chromatography (MonoQ column, 20 mM diethanolamine pH 8.4, NaCl gradient; for details, see §2.1.). The scFv MEM-57 eluted in two peaks that probably corresponded to the scFv monomer and dimer (data not shown). When each fraction was separately analyzed by SEC analysis, an equilibrium between the monomer, dimer and higher oligomers similar to that shown in Fig. 2(a) was observed.

Because the inhomogeneity of the protein originating from the equilibrium between scFv monomer and oligomers is not favourable for protein crystallization, we decided to screen for a buffer

Figure 2

Inhomogeneity of scFv MEM-57 in the original storage buffer (20 mM diethanolamine pH 8.4, 100 mM NaCl). (a) Size-exclusion chromatographic analysis of scFv MEM-57 at 7.5 mg ml⁻¹. Apparent molecular weights are indicated for each peak together with the percentage of the individual oligomeric forms in the total protein content. (b) The DLS particle-size distribution reveals polydispersity of scFv MEM-57 at 15 mg ml⁻¹.

composition which would stabilize the protein in one of the existing forms. The ThermoFluor assay (also known as differential scanning fluorimetry) is currently routinely used to screen for optimal protein storage buffer compositions for protein stability and conformational homogeneity (Ericsson *et al.*, 2006; Reinhard *et al.*, 2013). In our case, we not only used ThermoFluor to evaluate protein stability, but we also followed the protein oligomeric state, which was reflected by a specific shape of the melting curve. A screen consisting of 22 buffers at pH 4.5–8.5 was used in the presence or absence of 200 mM NaCl (Ericsson *et al.*, 2006). Conditions in which the protein exhibited a multiple-inflection melting curve, indicating the presence of multiple oligomeric forms, were excluded from further analysis of the melting temperature (T_m). In the T_m analysis, the buffer in which the protein exhibited a single-inflection curve with the highest T_m (331 K) was selected as the optimal protein storage buffer (100 mM sodium phosphate pH 7.5, 200 mM NaCl; see Fig. 3). The protein-unfolding curve for the original protein storage buffer also included a second inflection, which was more apparent in the negative first derivative of the curve as a second minimum.

SEC and DLS analyses were performed on the scFv MEM-57 protein in the optimized buffer. SEC confirmed an improved oligomeric homogeneity of the protein sample, as illustrated in Fig. 4(a). The equilibrium between the monomer and dimer has shifted significantly in the favour of the monomeric form; the monomer represented 88% of the total protein (compared with 65% of

monomer in the original storage buffer). DLS analysis revealed a monomodal particle-size distribution (Fig. 4b).

The positive effect of the optimized storage buffer composition on protein crystallizability was confirmed by the results of the initial crystallization trials; protein crystals were obtained in 41 out of 96 screened conditions (Fig. 5).

Selected crystals obtained in the screening diffracted to a maximal resolution of 6 Å (data not shown) when tested for X-ray diffraction at 120 K using a MAR345 image-plate system and a Nonius FR591 rotating-anode generator (Bruker Nonius B.V., Netherlands). Optimization of the diffraction quality of the crystals is currently in progress. This includes optimization of the crystallization conditions, cryoprotection optimization and optimization of the protein construct (specifically introducing cleavable tags at either the C-terminus or the N-terminus). The use of an epitope-derived peptide to stabilize the oligomeric state and/or antigen-binding site, a strategy successfully used previously for other scFv fragments (Rezácova *et al.*, 2001, 2005), cannot be used for our antibody fragment as the epitope of MEM-57 is not linear but structural.

4. Conclusions

Pre-crystallization analysis of protein samples using a combination of biochemical and biophysical methods such as size-exclusion chromatography, dynamic light scattering and the ThermoFluor assay can be used to help protein crystallization. We have employed these methods to optimize the stability and also the oligomeric homogeneity of scFv MEM-57 for crystallization. This approach resolved

Figure 3
ThermoFluor assay for scFv MEM-57. (a) ThermoFluor-based protein-unfolding curves for the original (black) and optimized (green) storage buffers are shown along with representative multiple-inflection curves which were excluded from further melting-temperature (T_m) analysis (blue). (b) Melting temperatures for storage buffers with the melting temperatures of other single-inflection buffers. The original and optimized storage buffers are highlighted in black and green, respectively.

Figure 4
Homogeneity of scFv MEM-57 in the optimized storage buffer (100 mM sodium phosphate pH 7.5, 200 mM NaCl). (a) Size-exclusion chromatographic analysis of scFv MEM-57 at 7.5 mg ml⁻¹. Apparent molecular weights are indicated for each peak together with the percentage of the individual oligomeric forms in the total protein content. The discrepancy in the apparent molecular weights for the scFv dimer in the original and optimized buffer (compare with Fig. 2a) might be caused by the regeneration and recalibration of the SEC column between these two runs. (b) The DLS particle-size distribution reveals monodispersity of scFv MEM-57 at 15 mg ml⁻¹.

Figure 5

Crystallization of scFv MEM-57 in the optimized storage buffer. (a) A scheme of the crystallization screening plate containing The PEGs Suite (Qiagen, Netherlands) is shown with the conditions yielding crystals highlighted in green. (b) An example of crystals obtained using 100 mM HEPES pH 7.5, 15% (w/v) PEG 20 000. (c) An example of crystals obtained using 200 mM sodium fluoride, 20% (w/v) PEG 3350. (d) An example of crystals obtained using 100 mM HEPES pH 7.5, 25% (w/v) PEG 3000.

the issue of unfavourable oligomerization of the scFv fragment by stabilization of the monomeric form and thus resulted in a protein sample with improved crystallizability.

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