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Preliminary X-ray analysis of a human V_H fragment at 1.8 Å resolution

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Small antibody fragments are more useful than full-size antibodies for achieving efficient biodistribution. As a first step towards the design of a clinically desirable antibody fragment, the crystallization of a human V_H fragment has been achieved. The fragment was derived from the single-chain antibody scFvM12, which recognizes a cancer-specific hypoglycosylated form of mucin. The V_H fragment was obtained by in-drop digestion of the scFvM12 with a low concentration of the broad-spectrum protease subtilisin Carlsberg. The crystal belongs to the monoclinic space group *C*2. The crystal diffracted to 1.8 Å resolution when analysed at 100 K using a rotating-anode X-ray generator.

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1. Introduction

Mucins are high-molecular-weight glycoproteins that occur as either secreted or membrane-spanning forms. Their function is to protect the apical surface of epithelia and they have been shown to be involved in cell–cell interactions, signalling and metastasis (Parry *et al.*, 2001). Human mucin-1 (MUC1) is a polymorphic type I transmembrane glycoprotein containing a variable number of 20 amino-acid tandem repeats PDTRPAPGSTAPPAHGVTSA (Hinoda & Imai, 1994). In normal cells, the extracellular MUC1 peptide core is covered with O-linked carbohydrate chains, whereas in rapidly growing cancer cells the protein core becomes exposed owing to aberrant glycosylation (Hilkens *et al.*, 1989), rendering the main immunodominant epitope PDTRP accessible. MUC1 is aberrantly overexpressed in most human carcinomas such as those of the breast, lung, colon, pancreas, prostate and ovary. The extracellular accessibility of the MUC1 polypeptide in cancer cells makes this glycoprotein a suitable immunotarget for diagnosis and therapy.

A large number of MUC1-specific monoclonal antibodies have been produced, but the stability, solubility and size of the antibody determines their effectiveness in cancer therapy. Smaller antibody fragments are advantageous over full-size antibodies, since small antigen-binding molecules can efficiently penetrate solid tumours (Mayer *et al.*, 1999).

After the discovery of functional heavy-chain antibodies in *Camelidae*, many attempts have been made to generate either humanized or human V_H fragments (Hamers-Casterman *et al.*, 1993), but non-specific interaction in the absence of the V_L domain often leads to their aggregation in solution, which suggests that further engineering is required to produce clinically useful reagents (Kortt *et al.*, 1995).

scFvM12 was chosen in comparison to other scFvs directed against MUC1 antigen on the basis of extensive validation through FACS, ELISA and immunofluorescence (Wong *et al.*, 2001). scFvM12 was constructed by assembling the V_H and V_L domains from two different human phage libraries (Sheets *et al.*, 1998). The V_L gene sequence of the scFvM12 is nearly identical to one of the germline V-genes, while the V_H gene has undergone multiple mutations; therefore, the V_H domain should dominantly contribute to the affinity of the scFvM12 towards the MUC1 antigen.

Although the structures of both murine or humanized Fab antibody fragments directed against MUC1 have been solved (Dokurno *et al.*, 1998), until now no attempt has been made to crystallize a human single antibody domain. Here, we report for the first time the crystallization and preliminary X-ray analysis of a human V_H fragment, a single-domain antibody obtained through limited proteolysis from an scFv.

2. Materials and methods

scFvM12 was expressed in *Escherichia coli* strain TG1 using a construct that targeted the recombinant protein to the periplasm. The protein was purified by immobilized metal-affinity chromatography (Wong *et al.*, 2001) and then dialyzed overnight against 30 m*M* sodium succinate pH 4.4, 20 m*M* NaCl, 2 m*M* ethylenediaminetetraacetic acid (EDTA) and 2 m*M* benzamidine–HCl. Further purification was carried out by strong cation-exchange chromatography using an Amersham Mono S HR 10/10 column. The recombinant antibody

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eluted at 90 m*M* NaCl using a linear NaCl gradient. The antibody was dialyzed overnight against 30 m*M* Tris pH 8.0, 20 m*M* NaCl, 2 m*M* EDTA and 2 m*M* benzamidine–HCl and then further purified by anion-exchange chromatography using an Amersham Mono Q HR 10/10 column. The recombinant antibody eluted at 50 m*M* NaCl using a linear NaCl gradient. The protein was concentrated and loaded onto an Amersham Superdex 75 Hi-Load 16/60 column. An isocratic elution was performed in 20 m*M* Tris pH 8.5 containing 20 m*M* NaCl, 0.5 m*M* EDTA and 1 m*M* dithiothreitol (DTT).

The protein concentration was determined at 280 nm by applying a theoretical molar extinction coefficient of 43 810 $mol^{-1} cm^{-1}$. The protein was concentrated to 13 $mg ml^{-1}$ and buffer-exchange was carried out using 20 m*M* Tris pH 7.5. The broad-spectrum serine protease subtilisin Carlsberg from *Bacillus licheniformis* (Sigma) was added to the scFvM12 solution in a 100:1 molar ratio (protein: enzyme) just prior to setting up the hanging-drop experiments.

After washing in reservoir solution, crystals were resolubilized in deionized water and subjected to matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) mass spectrometry (Tof Spec 2E, Micromass UK Limited) for mass determination and electrospray ionization tandem mass spectrometery (ESI–MS/MS) for sequence analysis (Q-Tof 2, Micromass UK Limited).

3. Results and discussion

It has been shown previously that proteins with high conformational heterogeneity (*e.g.* antibodies and kinases) are the most difficult to crystallize. Limited proteolysis can trim the flexible regions from such proteins and has been used to obtain well ordered crystals (Hoedemaeker *et al.*, 1997). We therefore used the broad-spectrum protease subtilisin Carlsberg to prepare well ordered and compact crystals from the recombinant scFvM12. Using the vapour-diffusion method and PEG 550 MME as a precipitating agent, crystals were grown at 277 and 290 K. For crystallization, the hanging drops consisted of 1.0 µl scFvM12/subtilisin mixture and 2.0 µl reservoir solution, *i.e.* 100 m*M* MES pH 6.5, 10 m*M* $ZnSO_4$, 25%(*v*/*v*) PEG 550 MME, 4 m*M* DTT and 0.04% NaN_3. Crystals appeared reproducibly after 5–10 d at 277 and 290 K. At 277 K the crystals were star-shaped, while at 290 K thin plates were observed. The morphological differences between the crystals prepared at different temperatures could be a consequence of differences in the kinetics of the protease-based digestion and the rate of drop supersaturation.

Table 1
Data-collection statistics.

Values in parentheses refer to the outer resolution shell.

Space group	*C*2
Unit-cell parameters (Å, °)	*a* = 72.01, *b* = 38.35, *c* = 37.57, $\alpha = \gamma = 90$, $\beta = 109.75$
Matthews coefficient ($Å^3 Da^{-1}$)	1.98
Solvent content (%)	38
Unit-cell volume ($Å^3$)	97652.034
No. molecules in AU	1
Observed reflections	46010
Unique reflections	8576
Wavelength (Å)	1.542
Resolution range (Å)	50.0–1.8 (1.86–1.80)
Completeness (%)	94.4 (87.8)
R_{merge}	0.031 (0.140)
$I/\sigma(I) > 2$ (%)	91.1 (77.0)

We found that the presence of $ZnSO_4$ in the mother liquor produced the best crystals. In the absence of Zn^{2+} we observed the formation of microcrystals, while other divalent metal ions (*e.g.* Mg^{2+}, Co^{2+} and Cd^{2+}) produced larger but morphologically defective crystals after 30–40 d. When the SO_4^{2-} ions were replaced by chloride ions, some synergistic effects were observed in improving the crystal quality but the crystal-growth rate was reduced to 30–40 d. The incubation time with subtilisin Carlsberg was the most critical factor influencing crystal development. It was found that crystals only appeared when the protease was mixed with the protein solution just prior to setting up the hanging drops. A longer pre-incubation period with the protease in the absence of a precipitating agent led to complete digestion of the scFvM12.

The thin plate crystals were mounted in small loops using glycerol as a cryoprotectant [final concentration 40%(*v*/*v*) in reservoir solution]. After mounting, the crystals were immediately flash-cooled to 100 K under a nitrogen jet (Oxford Cryosystems) and 125 frames ($\Delta\varphi = 1°$; three oscillations per frame; 30 min per image) were collected on a MAR 345 image plate using radiation from a Rigaku rotating copper-anode generator operated at 40 kV and 100 mA.

Using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997), the crystal was assigned to space group *C*2 with good data statistics (Table 1). The Matthews coefficient (Matthews, 1968) for scFvM12 (27.478 kDa) was calculated to be 0.89 $Å^3 Da^{-1}$. This value is considerably less than the normally accepted range. This was the first indication that the asymmetric unit might contain only one protein domain.

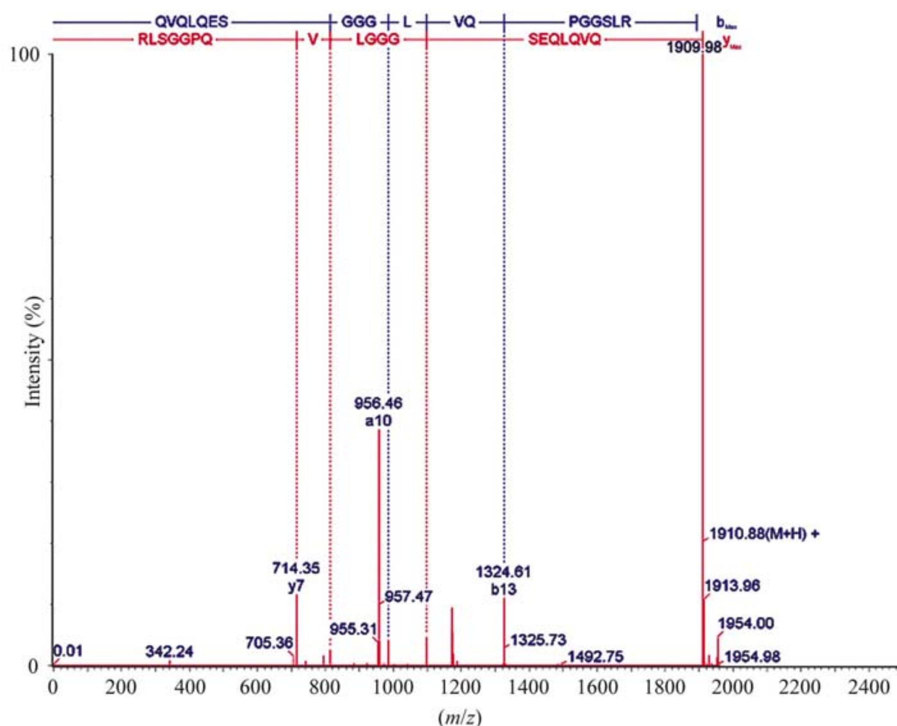

Figure 1
ESI–MS/MS spectrum of the N-terminal peptide generated after tryptic digestion of the resolubilized crystals. b_{Max} indicates the b-ions, which represent the N-terminal peptide sequence of the V_H domain of the scFvM12 antibody fragment.

To find out the content of the crystal, resolubilized crystals were analysed by MALDI–TOF mass spectrometry. The observed weight (12 346 Da) corresponded well to the theoretical molecular weight of the V_H domain (12 348 Da). Furthermore, the N-terminal peptide was clearly identified using electrospray ionization mass spectrometry (ESI-MS/MS) after tryptic digestion of the resolubilized crystals (Fig. 1). The sequence determination confirms the crystallization of only the V_H domain of the scFvM12 antibody.

Structure determination and refinement is in progress. The structure of the V_H fragment will be used as a basis to study the influence of camelization on structure–function properties and to derive a clinically desirable single-domain antibody of high affinity through structure-based antibody engineering.

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