Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

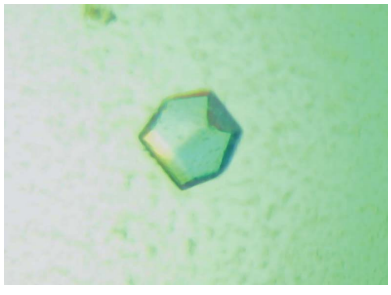
Production, purification, crystallization and preliminary X-ray diffraction analysis of the HIV-2-neutralizing V3 loop-specific Fab fragment 7C8

Hannes Uchtenhagen,[a] Samer Sourial,[a] Rosmarie Friemann,[b,c] Mariethe Ehnlund,[d] Anna-Lena Spetz,[a] Robert A. Harris,[e] Chaithanya Madhurantakam[a] and Adnane Achour[a]*

[a]Center for Infectious Medicine (CIM), F59 Department of Medicine, Karolinska University Hospital Huddinge, Karolinska Institutet, SE-141 86 Stockholm, Sweden, [b]Centre for Molecular Biology and Neuroscience (CMBN), Rikshospitalet University Hospital, N-0027 Oslo, Norway, [c]Department of Cell and Molecular Biology, Molecular Biophysics, Uppsala University Biomedical Center, SE-75 124 Uppsala, Sweden, [d]Infectious Diseases Unit, Department of Medicine, Karolinska University Hospital Solna, Karolinska Institutet, SE-171 76 Stockholm, Sweden, and [e]Neuroimmunology Unit, Department of Clinical Neurosciences, Karolinska University Hospital Solna, Karolinska Institutet, SE-171 76 Stockholm, Sweden

Correspondence e-mail: adnane.achour@ki.se

Received 30 March 2009
Accepted 31 May 2009



7C8 is a mouse monoclonal antibody that is specific for the third hypervariable loop (V3 loop) of the human immunodeficiency virus type 2 (HIV-2) associated protein gp125. Fab fragments of 7C8 effectively neutralize HIV-2. 7C8 was expressed and purified from a hybridoma cell line in order to establish the molecular basis underlying the specificity of the 7C8 antibody for the V3 loop as well as the specific role of the elongated third complementarity-determining region of the heavy chain (CDRH3). The antibody was digested with papain and Fab fragments were purified using size-exclusion chromatography. Hanging-drop vapour-diffusion crystallization techniques were employed and the protein was crystallized in 50 m*M* ammonium sulfate, 100 m*M* Tris–HCl pH 8.5, 25%(*w*/*v*) PEG 8000 and 2.5%(*w*/*v*) PEG 400 at 275 K. The analysed crystals belonged to the rhombohedral space group $P3_221$, with unit-cell parameters $a = b = 100.1$, $c = 196.8$ Å, and diffracted to 2.7 Å resolution.

1. Introduction

The surface glycoprotein unit (SU) of the human immunodeficiency virus (gp120 and gp125 in HIV-1 and HIV-2, respectively) is a major target for neutralizing antibodies and is thus considered to be of crucial importance for vaccine development. The third hypervariable loop (V3 loop) of gp125 and gp120 is essential for the function of the SU and is likely to have constrained flexibility (Hartley *et al.*, 2005). Accordingly, V3 loop-specific antibodies have been reported to inhibit viral infection (McKnight *et al.*, 1996; York *et al.*, 2001; Hartley *et al.*, 2005; Pantophlet & Burton, 2006), making it one of the few regions of the SU that is susceptible to neutralization (Hartley *et al.*, 2005; Pantophlet & Burton, 2006). A linear site localized at the crown of the V3 loop and composed of the four amino-acid residues GPGR has been identified as one of the main neutralizing determinants in HIV-1-associated gp120 (Javaherian *et al.*, 1989; Gorny *et al.*, 1992, 1993). Conversely, the HIV-2-associated gp125 has been described to harbour two highly conserved immunodominant motifs corresponding to the stretches of residues 315–318 (FHSQ) and 329–331 (WCR) localized within the central and C-terminal parts, respectively, of the V3 loop (Björling *et al.*, 1994).

Several three-dimensional structures of Fab fragments from antibodies specific for the V3 loop of HIV-1 have been determined either alone or in complex with V3 loop-derived peptides (Stanfield *et al.*, 1999, 2003; Rosen *et al.*, 2005). This has provided important insights into their mode of interaction with the V3 loop ligand as well as into the relative spatial disposition of the complementarity-determining regions (CDRs). A panel of HIV-2 V3 loop-specific murine monoclonal antibodies, some of which displayed neutralizing capacity, has previously been described in a functional setting (Björling *et al.*, 1994). The monoclonal antibody 7C8 binds with high specificity and efficiency to peptides containing the FHSQ epitope and its Fab fragments neutralize HIV-2 isolates (Björling *et al.*, 1994; Mörner *et al.*, 1999; Sourial *et al.*, 2006; Sourial & Nilsson, 2008). Interestingly, the third CDR of the heavy chain (CDRH3) of 7C8 is elongated when compared with the average length distribution in mouse antibodies (13 amino-acid residues *versus* an average of eight to nine; Wu *et al.*, 1993; Johnson & Wu, 1998; Sourial & Nilsson, 2008). This concords

with data from human neutralizing antibodies specific for gp120 and suggests an important role for the elongated CDRH3 loop, which is believed to be able to reach and overcome sterical hindrance, binding to potentially masked epitopes on the HIV envelope proteins (Johnson & Wu, 1998; Kwong *et al.*, 1998; Saphire *et al.*, 2001; Collis *et al.*, 2003; Ofek *et al.*, 2004; Stanfield *et al.*, 2004; Burton *et al.*, 2005).

In this study, the expression, purification and crystallization of the HIV-2-neutralizing Fab fragment of the antibody 7C8 are reported. A preliminary analysis of the X-ray diffraction data is also presented.

2. Materials and methods

2.1. Production and purification of the monoclonal antibody 7C8

Hybridoma cells expressing the murine monoclonal antibody 7C8 were grown in CELline 1000 membrane flasks (Integra Bioscience, Chur, Switzerland) using RPMI-1640 medium (Invitrogen, Paisley, Scotland) supplemented with MEM non-essential amino acids, L-glutamine, gentamycin (Invitrogen) and 15% foetal bovine serum (FBS; Invitrogen). Antibodies were purified using protein A affinity-column chromatography (HiTrap MabSelect, GE Healthcare, Little Chalfont, England) following the addition of 0.5 *M* trisodium citrate (Sigma, St Louis, Missouri, USA) to the clarified supernatant. The column was washed with 500 m*M* sodium citrate pH 8.5 and the antibody fraction was eluted with 0.1 *M* glycine (Sigma) at pH 2.7. The eluate was neutralized using Tris–HCl (Sigma) at pH 8.

2.2. Purification of 7C8 Fab fragment

Fab fragments of the monoclonal antibody 7C8 were prepared through limited digestion with papain (Sigma; Sourial & Nilsson, 2008). The reaction was carried out in 20 m*M* sodium phosphate buffer pH 7.4 containing 150 m*M* NaCl, 10 m*M* EDTA and 10 m*M* cysteine (Sigma). 50 mg papain was added per milligram of antibody and incubated for 1.5 h at 310 K. The reaction was terminated by adding iodoacetamide (Sigma) to a final concentration of 200 m*M* and the solution was dialysed against 20 m*M* Tris–HCl (Trizma, Sigma) pH 7.4 containing 150 m*M* NaCl (Sigma). Fab fragments were separated from intact IgG using a Superdex 75 10/300 size-exclusion column (GE Healthcare) at a flow rate of 0.5 ml min^{-1} and fractions were analysed using reducing SDS–PAGE and prestained molecular-weight markers (PageRuler Plus, Fermentas, St Leon-Rot, Germany). The purified Fab fragment was dialysed against 20 m*M* Tris–HCl pH 7.4 and concentrated to 10 mg ml^{-1} using an Amicon centrifugal filter with a 10 kDa cutoff (Millipore, Solna, Sweden).

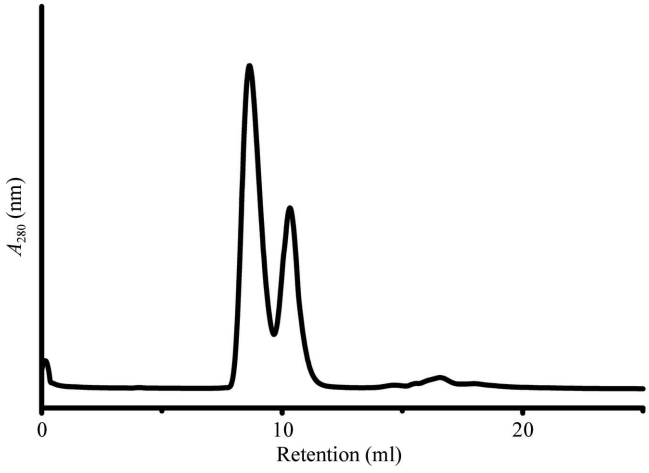


Figure 1
Size-exclusion chromatography of the purified murine antibody 7C8 following papain digestion. Preparative chromatography was carried out in 20 m*M* Tris–HCl pH 7.4 containing 150 m*M* NaCl using a Superdex 75 10/300 GL size-exclusion column with a flow rate of 0.5 ml min^{-1}. Peak 1 corresponds to the intact IgG and peak 2 corresponds to the neutralizing Fab fragment, with apparent molecular masses of about 150 and 50 kDa, respectively.

2.3. Crystallization and data collection

A brief survey of the Protein Data Bank for structures of Fab fragments that are either specific for HIV-1 gp120 (1nak, 1ggc, 2qsc, 1q1j, 2b0s, 1rzi) or share high sequence identity with the 7C8 Fab (1xf3, 3dif, 1ae6, 1xgy, 1i9j) did not indicate a common pattern for crystallization. Accordingly, preliminary crystallization conditions were screened using Crystal Screen and Crystal Screen 2 (Hampton Research, Aliso Viejo, California, USA) in 24-well Corning plates (Corning Incorporated, New York, USA) by hanging-drop vapour diffusion. Hanging drops containing 1 µl protein solution and 1 µl crystallization solution were equilibrated against reservoir containing 1 ml crystallization solution. For optimization of initial crystallization conditions, 2 µl protein solution and 2 µl crystallization solution were equilibrated in a hanging drop against 1 ml crystallization solution. 7C8 Fab crystals were cryoprotected using reservoir solution supplemented with 20%(*v*/*v*) glycerol and flash-cooled in a cold liquid-nitrogen stream. Diffraction data were collected at 100 K on beamline I711, MAX II, MAX-lab (Lund, Sweden) using a MAR CCD detector.

3. Results and discussion

3.1. Production and purification

The hybridoma cell line that expresses 7C8, classified as being of the IgG_1 subtype (Sourial & Nilsson, 2008), was originally isolated from mice immunized with a 15-mer peptide derived from the HIV-2 V3-loop region that includes the conserved stretch of residues FHSQ

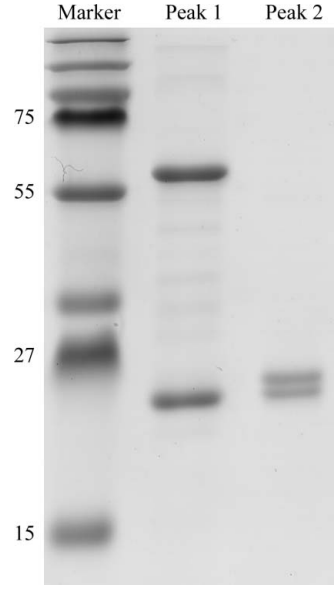


Figure 2
Analytical SDS–PAGE of the purified Fab fragment. Size-exclusion peak fractions 1 and 2 were analyzed on 12% SDS–PAGE under reducing conditions using a prestained molecular-weight marker (labelled in kDa). The two bands at 60 and 25 kDa in peak 1 correspond to the heavy and light chains of intact IgG. Peak 2 shows a doublet at about 25 kDa corresponding to the heavy-chain and light-chain fragments of the 7C8 Fab molecule.

(Björling *et al.*, 1994). This and subsequent studies established the binding of the purified antibody and derived Fab fragments to V3 loop-derived peptides and recombinant gp125, as well as the neutralization of the virus by the 7C8 Fab fragment (Björling *et al.*, 1994; Morner *et al.*, 1999; Sourial *et al.*, 2006; Sourial & Nilsson, 2008). The protein was expressed from the hybridoma cell line using membrane-based culture flasks, yielding up to 5 mg of antibody per 10 ml of cultured cells. Binding of the antibody to the recombinant protein A column could be improved by the addition of 500 m*M* trisodium citrate to the clarified culture supernatant. Papain digestion was conducted under strictly limiting conditions in order to avoid over-digestion of the antibody. The Fab fragment (peak 2) could be separated from intact antibody (peak 1) by size-exclusion chromatography (Fig. 1). Analysis of the pooled peak 2 fractions by SDS–PAGE under reducing conditions revealed a doublet at about 25 kDa characteristic of Fab fragments (the predicted molecular mass of the 7C8 Fab fragment is 47.2 kDa). Analysis of the fractions corresponding to peak 1 showed two bands at about 60 and 25 kDa, respectively, that correspond to the light and heavy chains of the intact IgG molecule (Fig. 2).

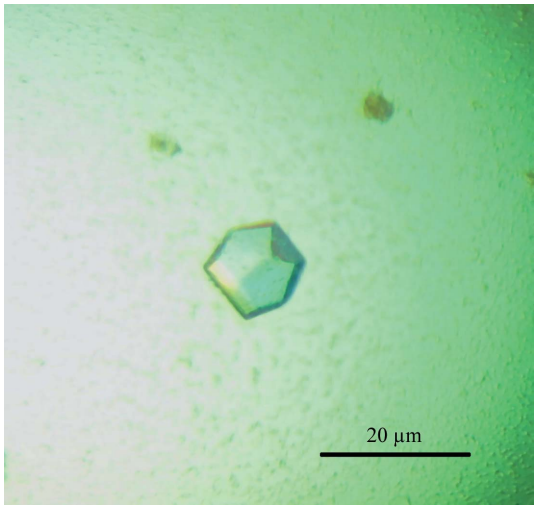


Figure 3
Picture of a representative 7C8 Fab crystal. Crystals appeared after two weeks in hanging drops in 100 m*M* Tris–HCl pH 8.5, 50 m*M* ammonium sulfate, 25%(*w*/*v*) PEG 8000 and 2.5%(*v*/*v*) PEG 400 at 275 K.

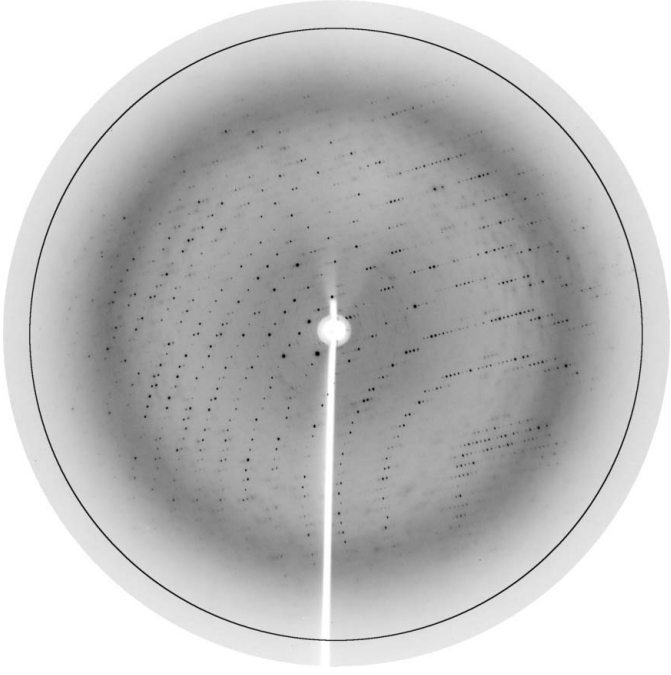


Figure 4
Representative diffraction pattern of the 7C8 Fab crystals. The ring indicates the outer limit of the highest resolution shell (2.7 Å).

Table 1
Statistics of data collection.

Values in parentheses are for the outer resolution shell.

Wavelength (Å)	1.6
Resolution (Å)	44.6–2.7 (2.85–2.7)
Space group	$P3_221$
Unit-cell parameters (Å)	$a = b = 100.1$, $c = 196.8$
V_M (Å^3 Da^{-1})	3.03
Solvent content (%)	59
No. of molecules in ASU	2
No. of observed reflections	245681 (31362)
No. of unique reflections	32045 (4588)
Redundancy	7.7 (6.8)
Completeness (%)	99.9 (99.9)
R_{merge}† (%)	7.0 (62.1)
$\langle I/\sigma(I)\rangle$	21.1 (2.3)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the weighted average intensity for all observations i of reflection hkl.

3.2. Crystallization

Crystals of the HIV-2-neutralizing Fab fragment 7C8 appeared initially in 0.2 *M* ammonium sulfate and 30%(*w*/*v*) PEG 8000. Further fine screening resulted in crystals of good quality (Fig. 3) that appeared after two weeks in 50 m*M* ammonium sulfate, 100 m*M* Tris–HCl pH 8.5, 25%(*w*/*v*) PEG 8000 and 2.5%(*v*/*v*) PEG 400 at 275 K.

3.3. X-ray diffraction study

The 7C8 crystals diffracted to a resolution of 2.7 Å and belonged to the rhombohedral space group $P3_221$, with unit-cell parameters $a = b = 100.1$, $c = 196.8$ Å. A representative diffraction pattern is displayed in Fig. 4. The diffraction data were indexed, integrated, scaled and merged using the programs *MOSFLM* (Leslie, 1992) and *SCALA* (Collaborative Computational Project, 1994). The Matthews coefficient was found to be 3.03 Å^3 Da^{-1}, suggesting the presence of two Fab molecules in the asymmetric unit with a solvent content of 59% (Matthews, 1968). Data-collection statistics are summarized in Table 1.

In conclusion, we have produced and purified the neutralizing antibody 7C8 specific for the V3-loop region of the HIV-2-associated protein gp125. Fab fragments of 7C8 were produced through papain digestion and purified. Crystallization conditions were determined and a diffraction data set was collected to 2.7 Å resolution. The determination of the crystal structure of the 7C8 Fab fragment will provide important insights into its specificity and the role of the extended CDRH3 in binding to the V3 loop-derived epitope.

We gratefully acknowledge access to synchrotron radiation at beamline ID14-1 at the ESRF laboratory, Grenoble, France, at beamline MX-41 at the BESSY laboratory, Berlin, Germany and at beamline 711 at MAX-lab, Lund University, Sweden. This study was financially supported by grants from the European Vaccines and Microbicides Enterprise (Europrise), the Swedish Medical Research Council and the Swedish National Board of Health and Welfare.

References

Björling, E., Chiodi, F., Utter, G. & Norrby, E. (1994). *J. Immunol.* **152**, 1952–1959.

Burton, D. R., Stanfield, R. L. & Wilson, I. A. (2005). *Proc. Natl Acad. Sci. USA*, **102**, 14943–14948.

Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.

Collis, A. V. J., Brouwer, A. P. & Martin, A. C. R. (2003). *J. Mol. Biol.* **325**, 337–354.

Gorny, M. K., Conley, A. J., Karwowska, S., Buchbinder, A., Xu, J. Y., Emini, E. A., Koenig, S. & Zolla-Pazner, S. (1992). *J. Virol.* **66**, 7538–7542.

Gorny, M. K., Xu, J. Y., Karwowska, S., Buchbinder, A. & Zolla-Pazner, S. (1993). *J. Immunol.* **150**, 635–643.

Hartley, O., Klasse, P. J., Sattentau, Q. J. & Moore, J. P. (2005). *AIDS Res. Hum. Retroviruses*, **21**, 171–189.

Javaherian, K., Langlois, A. J., McDanal, C., Ross, K. L., Eckler, L. I., Jellis, C. L., Profy, A. T., Rusche, J. R., Bolognesi, D. P., Putney, S. D. & Matthews, T. J. (1989). *Proc. Natl Acad. Sci. USA*, **86**, 6768–6772.

Johnson, G. & Wu, T. T. (1998). *Int. Immunol.* **10**, 1801–1805.

Kwong, P. D., Wyatt, R., Robinson, J., Sweet, R. W., Sodroski, J. & Hendrickson, W. A. (1998). *Nature (London)*, **393**, 648–659.

Leslie, A. G. W. (1992). *Jnt CCP4/ESF–EACBM Newsl. Protein Crystallogr.* **26**.

Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

McKnight, A., Shotton, C., Cordell, J., Jones, I., Simmons, G. & Clapham, P. R. (1996). *J. Virol.* **70**, 4598–4606.

Mörner, A., Achour, A., Norin, M., Thorstensson, R. & Björling, E. (1999). *Virus. Res.* **59**, 49–60.

Ofek, G., Tang, M., Sambor, A., Katinger, H., Mascola, J. R., Wyatt, R. & Kwong, P. D. (2004). *J. Virol.* **78**, 10724–10737.

Pantophlet, R. & Burton, D. R. (2006). *Annu. Rev. Immunol.* **24**, 739–769.

Rosen, O., Chill, J., Sharon, M., Kessler, N., Mester, B., Zolla-Pazner, S. & Anglister, J. (2005). *Biochemistry*, **44**, 7250–7258.

Saphire, E. O., Parren, P., Pantophlet, R., Zwick, M. B., Morris, G. M., Rudd, P. M., Dwek, R. A., Stanfield, R. L., Burton, D. R. & Wilson, I. A. (2001). *Science*, **293**, 1155–1159.

Sourial, S. & Nilsson, C. (2008). *Virol. J.* **5**, 96.

Sourial, S., Nilsson, C., Warnmark, A., Achour, A. & Harris, R. A. (2006). *Curr. HIV Res.* **4**, 229–237.

Stanfield, R., Cabezas, E., Satterthwait, A., Stura, E., Profy, A. & Wilson, I. (1999). *Structure*, **7**, 131–142.

Stanfield, R. L., Ghiara, J. B., Ollmann Saphire, E., Profy, A. T. & Wilson, I. A. (2003). *Virology*, **315**, 159–173.

Stanfield, R. L., Gorny, M. K., Williams, C., Zolla-Pazner, S. & Wilson, I. A. (2004). *Structure*, **12**, 193–204.

Wu, T. T., Johnson, G. & Kabat, E. A. (1993). *Proteins*, **16**, 1–7.

York, J., Follis, K. E., Trahey, M., Nyambi, P. N., Zolla-Pazner, S. & Nunberg, J. H. (2001). *J. Virol.* **75**, 2741–2752.