

Xiaodong Zhao,^{a,b} Ming Li,^{a,b}
Yanhui Xu,^{a,b} Zhiyong Lou,^{a,b}
Zhaohui Meng,^{a,b} Shu Li,^c
Bo Tian,^d George F. Gao^d and
Zihe Rao^{a,b*}

^aLaboratory of Structural Biology, Tsinghua University, Beijing 100084, People's Republic of China, ^bNational Laboratory of Macromolecules, Institute of Biophysics, Chinese Academy of Science, Beijing 100101, People's Republic of China, ^cSchool of Life Science, Wuhan University, Wuhan, People's Republic of China, and ^dInstitute of Microbiology, Chinese Academy of Sciences, Beijing 100080, People's Republic of China

Correspondence e-mail:
raozh@xtal.tsinghua.edu.cn

Received 13 October 2004
Accepted 20 January 2005
Online 1 February 2005

Cloning, expression, purification, crystallization and preliminary crystallographic study of the protein module (BIV2-Helix) in the fusion core of bovine immunodeficiency-like virus (BIV) gp40

The fusion core of bovine immunodeficiency virus (BIV) gp40 is proposed to be involved in membrane fusion. However, no crystal structures are yet available. A predicted protein module BIV2-Helix of BIVgp40 has been expressed in *Escherichia coli* and purified by chromatography. Recombinant BIV2-Helix was crystallized using the hanging-drop vapour-diffusion technique at 291 K. The crystals were grown in MPD and belonged to the primitive rhombohedral space group *R*3, with unit-cell parameters $a = 39.17$, $b = 39.17$, $c = 295.05$ Å and two molecules per asymmetric unit. X-ray diffraction data were collected to 1.76 Å in the home laboratory from a single crystal.

1. Introduction

Retroviruses are responsible for a number of diseases in humans and other animals (Gonda *et al.*, 1989; Haase, 1986). The lentivirus subfamily of retroviruses includes the agents of AIDS, human immunodeficiency virus types 1 (HIV-1) and 2 (HIV-2) (Barre-Sinoussi *et al.*, 1983; Clavel *et al.*, 1986; Gallo *et al.*, 1984), as well as the simian immunodeficiency virus (Daniel *et al.*, 1985; Kanki *et al.*, 1985), visna virus (Sigurdsson & Palsson, 1958), ovine progressive pneumonia virus (Cutlip & Laird, 1976; Kennedy *et al.*, 1968), caprine arthritis encephalitis virus (Crawford *et al.*, 1980), feline immunodeficiency virus (Pedersen *et al.*, 1987), equine infectious anaemia virus (McGuire *et al.*, 1987) and bovine immunodeficiency-like virus (BIV; Braun *et al.*, 1988; Gonda *et al.*, 1987; Van Der Maaten *et al.*, 1972). BIV resembles HIV-1 in many aspects of its pathogenesis, ultrastructure, genome organization and infectious cycle in culture (Braun *et al.*, 1988; Garvey *et al.*, 1990). Like HIV or other enveloped animal virus, BIV must enter a host cell by fusing its own membrane coat with that of the cell to release its contents. These membrane-fusion events are mediated by specific proteins, called fusion cores, on the viral membrane (Xu, 2004). The prototypes of these fusion-core proteins from influenza haemagglutinin (HA) and HIV-1 envelope protein (Env) gp41 share common structural properties, namely a coiled-coil six-helix bundle in the post-fusion state (Ecker & Kim, 2001). Two proteins of HIV-1, the transmembrane subunit gp41 and the surface protein gp120, are responsible for virus fusion and entry into host cells. gp120 initiates infection by binding to the cellular receptor CD4 and co-receptor, and gp40 mediates the actual virus-cell membrane fusion process (Kwong *et al.*, 1998; Turner & Summers, 1999; Sattentau, 1998; Wyatt *et al.*, 1998). In attachment and membrane-fusion processes, gp41 undergoes a series of conformational changes during which the virus enters into the host cells. However, the structure and function of the respective partners of gp62 and gp40 in BIV are poorly understood. Previous studies have revealed that some protein modules are crucial for membrane-viral or membrane-membrane fusion (Xu, 2004; Xu, Gao *et al.*, 2004; Xu, Liu *et al.*, 2004). Among them, a predicted protein module (BIV2-Helix) is highly hydrophobic and is believed to play an important role in directing insertion of BIV gp40 into the cellular lipid membrane. In this study, two highly conserved heptad-repeat (HR) regions, HR1 (residues 580–630) and HR2 (residues 667–694), acting as scaffolding modules in gp40 have been characterized using a computer program called *LearnCoil-VMF* (Singh *et al.*, 1999). We studied the interactions between HR1 and HR2 of BIV gp40 by structural approaches, in the hope that it will reveal some clues to the



mechanism of BIV gp40-induced membrane fusion during host-cell entry. This may open a potential new avenue to the development of new peptide inhibitors against BIV.

2. Expression and purification

The α -helix structure of the two heptad-repeat regions, HR1 (residues 580–630) and HR2 (residues 667–694), of BIVgp40 (GenBank Accession No. NP_040566) was predicted by the *LearnCoil-VMF* program (<http://learncoil-vmf.lcs.mit.edu/cgi-bin/vmf>), which was specifically developed for the identification of potential coiled-coil heptad-repeat regions in viral fusion proteins (Singh *et al.*, 1999). The construct of HR1 and HR2 connected by a linker SGGRGG was generated by the PCR approach and confirmed by sequencing. The PCR products were inserted into the multi-clone site *Bam*HI/*Xho*I of the expression vector pGEX-6p-1. Recombinant BIV2-Helix was expressed in *Escherichia coli* strain BL21(DE3); the cells were induced with 0.2 mM IPTG at 291 K overnight. After cell lysis, the cellular debris was removed by centrifugation. The supernatant from the cell lysis was applied onto a GST column pre-equilibrated with phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ pH 7.3) and the column was washed with

PBS buffer until no protein was detectable in the eluate. The GST-fused BIV2-Helix protein was incubated with 1.5 μ M 3C proteinase at room temperature for 2 h. The eluted protein was concentrated and applied onto a Superdex G75 column pre-equilibrated with PBS. The main peak of elution from the column was collected, exchanged into a new buffer (10 mM MES pH 6.0, 15 mM NaCl) and concentrated to 5 mg ml⁻¹ by ultrafiltration.

3. Crystallization of BIV2-Helix

Drops (2 μ l protein solution mixed with 2 μ l mother liquor) were allowed to equilibrate against 500 μ l mother liquor. Hampton Research crystallization kits were used for initial screening and the experiments were carried out at temperature of 291 K. Initial crystals were obtained in eight conditions after initial screening, but were small and diffracted very poorly (Fig. 1). Screening by varying the pH values and the concentration of the precipitants PEG, 2-propanol and MPD improved the crystal size and the diffraction quality. Colourless hexagonal crystals were obtained using the condition 0.1 M Tris pH 8.5, 0.2 M ammonium dihydrogen phosphate, 50% (v/v) MPD (2-methyl-2,4-pentanediol) after 4 d at 291 K (Fig. 2), with dimensions 0.05 \times 0.05 \times 0.02 mm.

4. Data collection and processing

Data collection from BIV2-Helix was performed on a Rigaku RU2000 rotating copper-anode X-ray generator operated at 48 kV and 98 mA (Cu K α ; λ = 1.5418 Å) with a MAR 345 image-plate detector. The crystal was mounted in a nylon loop and flash-cooled in a cold nitrogen-gas stream at 100 K using an Oxford Cryosystems cryocooler with reservoir solution as the cryoprotectant. The oscillation range, exposure time and crystal-to-film distance were 1° per frame, 5 min per frame and 120 mm, respectively. A diffraction image is shown in Fig. 3. Image data were processed using the program



Figure 1
An initial poorly diffracting crystal.



Figure 2
A typical crystal grown following optimization of the crystallization parameters. The size of the crystal is approximately 0.05 \times 0.05 \times 0.02 mm.

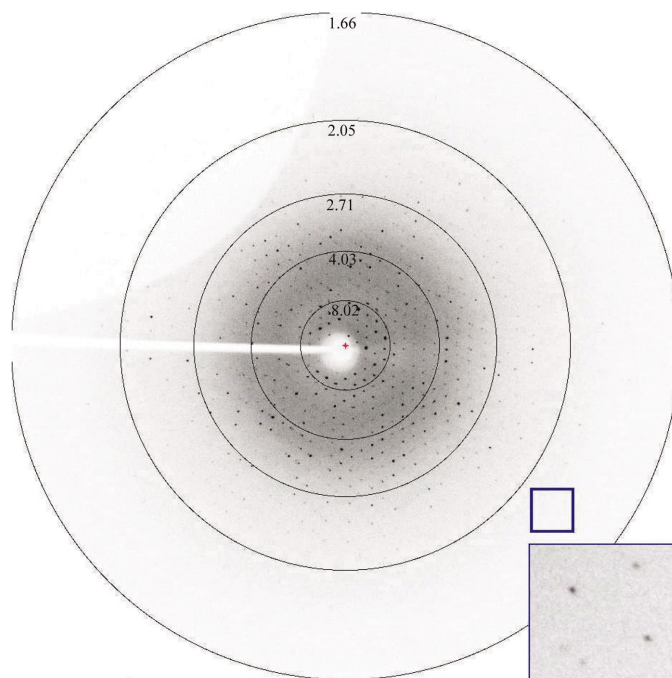


Figure 3
A typical X-ray diffraction pattern. The resolution at the edge of the image plate is 1.66 Å.

Table 1

Data-collection and processing statistics.

Values in parentheses correspond to the highest resolution shell.

Space group	R3
Unit-cell parameters (Å)	
<i>a</i> = <i>b</i> (Å)	39.17
<i>c</i> (Å)	295.05
Wavelength (Å)	1.5418
Resolution range (Å)	50–1.76
Observed reflections	81548
Unique reflections	18122
Completeness (%)	98.8
$\langle I/\sigma(I) \rangle$	22.2 (1.2)
$R_{\text{merge}}^{\dagger}$ (%)	5.4 (54.3)

$\dagger R_{\text{merge}} = \sum_h \sum_l |I_{h,l} - \bar{I}_h| / \sum_h \sum_l I_{h,l}$ for the intensity *I* of *i* observations of reflection *h*.

packages *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). Data statistics are listed in Table 1.

5. Results and discussion

BIV2-Helix can be crystallized under several conditions. However, the optimum quality crystals were obtained in 50% MPD, 0.1 *M* Tris pH 8.5, 0.2 *M* ammonium dihydrogen phosphate at 291 K. The crystals belong to space group *R3*, with unit-cell parameters *a* = 39.17, *b* = 39.17, *c* = 295.05 Å, $\alpha = 90$, $\beta = 90$, $\gamma = 120^\circ$. The data were 98.8% complete to 1.76 Å resolution. Based on the molecular weight of BIV2-Helix (10 kDa) and the space group *R3* it was assumed that each crystal contains two molecules per asymmetric unit. The solvent content is calculated to be 43.3% and the Matthews coefficient (V_M) is 2.2 Å³ Da⁻¹. A single point-mutant construct has been generated for expression of a selenomethionine-derivative protein. Crystallization of this construct is now in progress. This part of the work, together with the subsequent structural and functional analysis, will be published elsewhere.

We are grateful to Mr Fei Sun for helpful advice and discussion. This work was supported by the State '863' High-Tech Project (Grant No. 2002BA711A12), '973' Project (Grant No. G1999075602) and NSFC Grant No. 30221003.

References

- Barre-Sinoussi, F., Chermann, J. C., Rey, F., Nugeyre, M. T., Chamaret, S., Gruest, J., Dautuet, C., Axler-Blin, C., Vezinet-Brun, F., Rouzioux, C., Rozenbaum, W. & Montagnier, L. (1983). *Science*, **220**, 868–871.
- Braun, M. J., Lahn, S., Boyd, A. L., Kost, T. A., Nagashima, K. & Gonda, M. A. (1988). *Virology*, **167**, 515–523.
- Clavel, F., Guetard, D., Brunt-Vezint, F., Chamaret, S., Rey, M.-A., Santos-Ferreira, M. O., Laurent, A. G., Dautuet, C., Katlama, C., Rouzioux, C., Klatzmann, D., Champalimaud, J. L. & Montagnier, L. (1986). *Science*, **233**, 343–346.
- Crawford, T. B., Adams, D. S., Cheevers, W. P. & Cork, L. C. (1980). *Science*, **207**, 997–999.
- Cutlip, R. C. & Laird, G. A. (1976). *Am. J. Vet. Res.* **37**, 1377–1382.
- Daniel, M. D., Letvin, N. L., King, N. W., Kannagi, M., Sehgal, P. K., Hunt, R. D., Kanki, P. J. & Desrosiers, R. C. (1985). *Science*, **228**, 1201–1204.
- Ecker, D. M. & Kim, P. S. (2001). *Annu. Rev. Biochem.* **70**, 777–810.
- Gallo, R. C., Salahuddin, S. Z., Popovic, M., Shearer, G. M., Kaplan, M., Haynes, B. F., Palker, T. J. & Markham, P. D. (1984). *Science*, **224**, 500–503.
- Garvey, K. J., Oberste, M. S., Elser, J. E., Braun, M. J. & Gonda, M. A. (1990). *Virology*, **175**, 391–409.
- Gonda, M. A., Boyd, A. L., Nagashima, K. & Gilden, R. V. (1989). *Arch. AIDS Res.* **3**, 1–42.
- Gonda, M. A., Braun, M. J., Carter, S. G., Kost, T. A., Bess, J. W. Jr, Arthur, L. O. & Van Der Maaten, M. J. (1987). *Nature (London)*, **330**, 388–391.
- Haase, A. T. (1986). *Nature (London)*, **322**, 130–136.
- Kanki, P. J., McLane, M. F., King, N. W. Jr, Letvin, N. L., Hunt, R. D., Sehgal, P., Daniel, M. D. & Essex, M. (1985). *Science*, **228**, 1199–1201.
- Kennedy, R. C., Eklund, C. M., Lopez, C. & Hadlow, W. J. (1968). *Virology*, **35**, 483–484.
- Kwong, P. D., Wyatt, R., Robinson, J., Sweet, R. W., Sodroski, J. & Hendrickson, W. A. (1998). *Nature (London)*, **393**, 648–659.
- McGuire, T. C., O'Rourke, K. & Cheevers, W. P. (1987). *Contrib. Microbiol. Immunol.* **8**, 77–89.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Pedersen, N. C., Ho, E. N., Brown, M. L. & Yamamoto, J. K. (1987). *Science*, **235**, 790–793.
- Sattentau, Q. J. (1998). *Structure*, **6**, 945–949.
- Sigurdsson, B. & Palsson, P. A. (1958). *Br. J. Exp. Pathol.* **39**, 519–528.
- Singh, M., Berger, B. & Kim, P. S. (1999). *J. Mol. Biol.* **290**, 1031–1041.
- Turner, B. G. & Summers, M. F. (1999). *J. Mol. Biol.* **285**, 1–32.
- Van Der Maaten, M. J., Boothe, A. D. & Seger, C. L. (1972). *J. Natl Cancer Inst.* **49**, 1649–1657.
- Wyatt, R., Kwong P. D., Desjardins, E., Sweet, R. W., Robinson, J., Hendrickson, W. A. & Sodroski, J. G. (1998). *Nature (London)*, **393**, 705–711.
- Xu, Y. (2004). PhD thesis. Tsinghua University, People's Republic of China.
- Xu, Y., Gao, S., Cole, D. K., Zhu, J., Su, N., Wang, H., Gao, G. F. & Rao, Z. (2004). *Biochem. Biophys. Res. Commun.* **315**, 664–670.
- Xu, Y., Liu, Y., Lou, Z., Qin, L., Li, X., Bai, Z., Pang, H., Tien, P., Gao, G. F. & Rao, Z. (2004). *J. Biol. Chem.* **279**, 30514–30522.