Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Purification, crystallization and preliminary X-ray analysis of Triatoma virus (TrV) from *Triatoma infestans*

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Received 2 June 2004
Accepted 21 June 2004

Triatoma virus (TrV) is a viral pathogen of the blood-sucking reduviid bug *Triatoma infestans*, the most important vector of American human trypanosomiasis (Chagas' disease). TrV has been putatively classified as a member of the Cripavirus genus (type cricket paralysis virus) in the *Dicistroviridae* family. This work describes the purification of TrV particles from infected *T. infestans* and their crystallization and preliminary crystallographic analyses. Two different crystal forms, rhombohedral and orthorhombic, were obtained at room temperature by the hanging-drop vapour-diffusion technique using polyethylene glycol and polyethylene glycol monomethylether as precipitants. The rhombohedral crystals have unit-cell parameters $a = b = 306.6$, $c = 788.4$ Å (hexagonal setting), diffract to 3.2 Å resolution and contain one-third of the viral particle per asymmetric unit. The orthorhombic crystals have cell parameters $a = 336$, $b = 351$, $c = 332$ Å, diffract to about 2.5 Å resolution, and contain one-half of a virus particle in the asymmetric unit. A complete diffraction data set has been collected to 3.2 Å resolution, using synchrotron radiation, from a single rhombohedral crystal under cryogenic conditions.

1. Introduction

American human trypanosomiasis or Chagas' disease is a human illness with a wide distribution in central and South America, is endemic in 21 countries, and results in 16–18 million persons infected and 100 million people at risk (WHO, 1999). The disease is caused by the flagellated protozoan parasite *Trypanosoma cruzi*, which is transmitted to humans by blood-sucking reduviid bugs, *i.e.* triatomines. Chagas' disease has an acute stage characterized by fever, swelling of lymph glands, enlargement of the liver and spleen, or local inflammation at the site of infection. Chronic forms develop some 10–20 years after infection, causing irreversible damage to the heart, oesophagus and colon, with dilatation and disorders of nerve conduction of these organs. Patients with severe chronic disease ultimately die, usually from heart failure. There is, at present, no effective treatment for such cases (WHO, 1999).

Triatoma virus (TrV) is a viral pathogen of *Triatoma infestans* (Muscio *et al.*, 2000; Rozas-Dennis & Cazzaniga, 2000), the main vector of *T. cruzi*. Both transovarian and horizontal mechanisms are involved in the transmission of TrV among triatomines (Muscio, 1988; Muscio *et al.*, 1997, 2000; Rozas-Dennis *et al.*, 2002). Some of the pathological effects of this virus observed in colonies of triatomines are a high mortality rate, delayed development and reduction in fecundity (Rozas-Dennis & Cazzaniga, 2000). TrV was suggested as a potential control agent against triatomines (Czibener *et al.*, 2000, and references therein) and thereafter for the spread of Chagas' disease. On the basis of its biophysical properties, TrV was initially referred to as a picorna-like virus (Muscio *et al.*, 1988). However, after the TrV genome was fully sequenced (Czibener *et al.*, 2000), the virus was tentatively reclassified as a member of the Cripavirus genus in the family *Dicistroviridae* (Mayo, 2002).

The TrV virion consists of a non-enveloped capsid that encloses the viral genome, a molecule of positive-strand RNA of 9010 nucleotides. The capsid is formed of 60 protomers, each of which is believed to contain four structural proteins, VP1 (39 kDa), VP2 (37 kDa), VP3 (33 kDa) and a minor one, VP4 (45 kDa) (Czibener *et al.*, 2000). The protomers self-assemble to form a $T = 1$ (pseudo-triangulation $p = 3$) icosahedron of about 30 nm diameter.

2. Materials and methods

2.1. Virus purification

TrV was purified from infected triatomines, essentially following the procedure described by Rozas-Dennis *et al.* (2002), with minor modifications. Whole bodies of ten (adults and

fifth-instar nymphs) frozen (255 K) dead specimens after experimental infection of *T. infestans* (Rozas-Dennis *et al.*, 2002) were macerated in 10 ml of a 1:1 mix of NMT buffer (10 m*M* NaCl, 1 m*M* $MgCl_2$, 50 m*M* Tris–HCl pH 7.5) and chloroform. The homogenate was filtered and centrifuged at $4400g_{av}$ for 20 min at 277 K. The supernatant solution was centrifuged at $35\,000g_{av}$ and 277 K for 150 min, and the resultant pellet was resuspended in 4 ml of NMT buffer plus 1%(*w*/*v*) *N*-lauryl sarcosine; CsCl (2.3 g) was then added to form a 36%(*w*/*v*) final concentration (density 1.349 g cm^{-3}). A CsCl gradient was formed following centrifugation at $116\,000g_{av}$ overnight at 277 K. The resulting virus band was collected with a peristaltic pump (Miniplus 3 Gilson) and dialysed against 500 m*M* KCl, 1 m*M* $MgCl_2$ and 50 m*M* Tris–HCl pH 7.5. The integrity of the viral particles was checked by electron microscopy (Fig. 1).

2.2. Crystallization

TrV was crystallized at room temperature using the hanging-drop vapour-diffusion method. The initial crystallization screening was performed with Hampton Research Crystal Screens (Laguna Niguel, CA, USA). The virion concentration was 1.4 mg ml^{-1} in 50 m*M* Tris–HCl pH 7.5, 500 m*M* KCl and 1 m*M* $MgCl_2$. Typically, 1 µl of virion solution was mixed with an equal volume of a reservoir solution. The preliminary crystallization conditions were optimized by variation of polyethylene glycol (PEG) and polyethylene glycol monomethylether (PEG–MME) size (1500–8000), and by adding salt to the reservoir (500 m*M* of NaCl, NaBr or KCl). Crystals (type I) were obtained with 13%(*w*/*v*) PEG–MME 2000 in 100 m*M* Tris–HCl pH 8.5 and the addition of 500 m*M* KCl to the reservoir before equilibration. These crystals appeared overnight, and after five days they reached dimensions of 150 × 100 × 80 µm (Fig. 2*a*). Recently, a second crystal form (type II) was obtained in 6%(*w*/*v*) PEG 8000, 100 m*M* sodium citrate pH 5.6, 5%(*v*/*v*) 2-propanol and 500 m*M* NaCl (Table 1 and Fig. 2*b*). Preliminary diffraction experiments with these crystals showed diffraction up to 2.5 Å resolution.

2.3. Data collection, processing and analysis

TrV rhombohedral crystals (type I) were cryoprotected by soaking for 5 min in 20%(*w*/*v*) PEG–MME 2000 in 100 m*M* Tris pH 8.5, 500 m*M* KCl and 30%(*v*/*v*) glycerol, and then cryocooling by plunging into liquid ethane. Diffraction data were collected at 100 K using a nitrogen stream on a quantum ADSC Q4R CCD detector, using synchrotron radiation at the ESRF beamline ID14-1, Grenoble. 200 frames, each with an oscillation range of 0.3° (Fig. 3*a*), were collected, making a total of 60°.

TrV orthorhombic crystals (type II) were cryoprotected by soaking for 5 min in 15%(*w*/*v*) PEG 8000, in 100 m*M* sodium citrate pH 5.6, 500 m*M* NaCl, 5%(*v*/*v*) 2-propanol and 28%(*v*/*v*) glycerol, and then cryocooling by plunging into liquid ethane. Diffraction data were collected at 100 K using a nitrogen stream on a MARMOSAIC CCD detector, using synchrotron radiation at the ESRF beamline ID23-1, Grenoble. A total of 245 frames, each with an oscillation range of 0.25° (Fig. 3*b*), were collected, covering 61.25°. However, the diffraction power of the crystals decayed quickly, and only the first 15 images display measurable data to 2.5 Å resolution.

Determination of the unit cell and space group, and the integration of the diffraction data, were performed using *MOSFLM* (Leslie, 1992). The data were scaled and merged, and the space group was assigned, with *SCALA* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). Self-rotation function maps were calculated using *MOLREP* from the *CCP*4 suite (Vagin & Teplyakov, 1997), calculated with spherical angles of κ = 72, 120 and 180° for five-, three- and twofold symmetry axes (Fig. 4). Strong peaks were observed for each section corresponding to six fivefold axes, ten threefold symmetry axes including the crystallographic threefold axis, and 15 twofold symmetry axes, accounting for the icosahedral symmetry of the TrV capsid.

Table 1
TrV crystal parameters and crystallization conditions.

Crystal	Space group	Resolution (Å)	Unit-cell parameters (Å, °)	Virions per cell	Crystallization conditions
Type I	*H*3	3.2	$a = b = 306.6$, $c = 788.4$, $\alpha = \beta = 90$, $\gamma = 120$	3	13% PEG–MME 2000, 100 m*M* Tris–HCl pH 8.5, 500 m*M* KCl
Type II†	$P2_12_12$	2.5	$a = 336$, $b = 351$, $c = 332$, $\alpha = \beta = \gamma = 90$	2	6% PEG 8000, 100 m*M* sodium citrate pH 5.6, 5% 2-propanol, 500 m*M* NaCl

† Completeness of the data set is only to 60%. The measured systematic absences and the unit-cell content are in agreement with the space group $P2_12_12$. A complete data set is necessary in order to confirm these parameters.

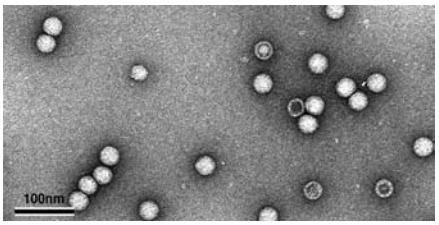

Figure 1
35000X TrV particles [stained with 1%(*w*/*v*) uranyl acetate]. The micrograph was recorded with a Phillips CM12 electron microscope operated at 80 kV. Film Kodak SO163 Image Plate, 5 min in Kodak D19 developer. Bar = 100 mm.

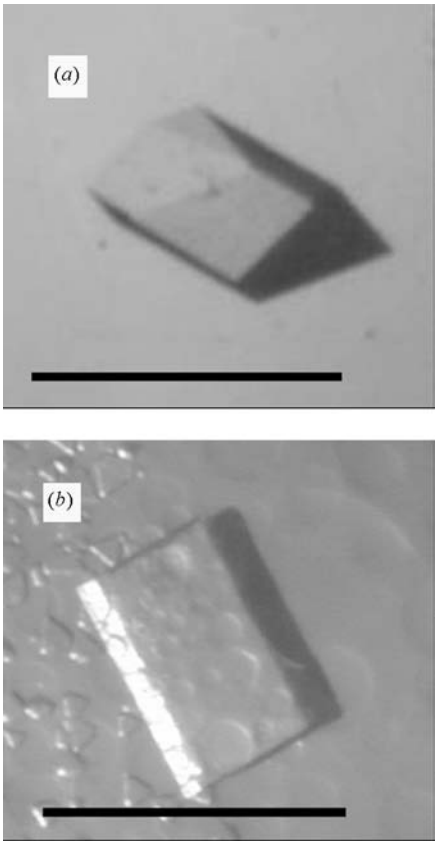

Figure 2
(*a*) Photograph of TrV rhombohedral (H3) crystals (type I). Bar 200 µm. (*b*) Photograph of TrV orthorhombic crystals (type II). Bar 200 µm.

3. Results and discussion

A complete 3.2 Å data set was collected from a single type I crystal. The unit cell was consistent with a rhombohedral space group with unit-cell parameters $a = b = 306.6$, $c = 788.4$ Å (hexagonal setting) and $a = b = c = 317.3$ Å, $\alpha = \beta = \gamma = 58.4°$ (rhombohedral setting). Scaling and merging of the data revealed the space group to be *H*3, with a crystal packing such that one-third of a virus particle is present in the crystal asymmetric

unit, *i.e.* with an icosahedral threefold axis coincident with a crystallographic threefold axis. The hexagonal unit cell therefore contains three viral particles, and the primitive rhombohedral unit cell contains one full particle. The solvent content is about 67% of the unit-cell volume. The statistics of the diffraction data processing are provided in Table 2. A molecular replacement (MR) solution was obtained using program *AMoRe* (Navaza, 1994), using as a starting model the coordinates of cricket paralysis virus (CrPV, PDB code 1b35) converted into a polyalanine chain. Despite the poor sequence identity between TrV and CrPV (26%), a clear solution was obtained, with an *R* factor of 45.1% and a correlation coefficient (CC) of 30% with data in the resolution range 25–8 Å. This solution detached clearly from the other putative MR solutions, the second best having an *R* factor of 48.5% and a CC of 21%. The atomic model of the TrV capsid is being built on the basis of the initial MR solution, currently improved by real-space averaging using the program *DM* (Cowtan & Main, 1993), taking advantage of the 20-fold redundancy present in the asymmetric unit. A partial diffraction data set was collected from one type II crystal (orthorhombic) to 2.5 Å resolution. Synchrotron beam-time is already scheduled to collect a full data set, which will allow full refinement of the atomic model of the virus particle. Given the radiation sensitivity of type II crystals, several crystals will be needed for collecting a complete high-resolution data set.

Table 2
TrV rhombohedral crystal (type I) data-collection statistics.

Values in parentheses refer to the highest-resolution shell.

Number of crystals	1
Temperature (K)	100
Space group	Rhombohedral (*H*3)
Cell parameters (Å, °)	$a = b = 306.6$, $c = 788.4$, $\alpha = \beta = 90$, $\gamma = 120$
Wavelength (Å)	0.93
Resolution range (Å)	25–3.2 (3.37–3.2)
Reflections	1 109 595 (130 534)
Unique reflections	453 290 (65 678)
Completeness (%)	99.2 (94.4)
Redundancy	2.4 (2)
$\langle I/\sigma_{(I)}\rangle$	4.3 (2)
$R_{\rm sym}$ (%)	12.2 (35.9)

4. Conclusions

The diffraction data collected on the rhombohedral (type I) crystals of TrV reported in this work provide enough information for

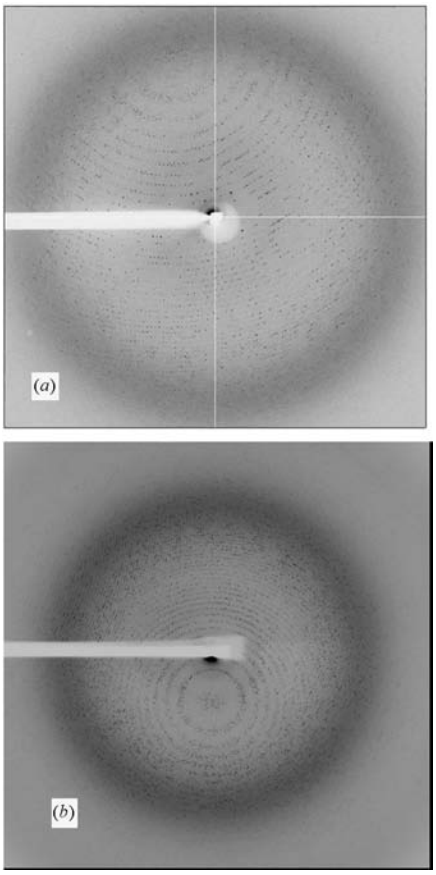

Figure 3
(*a*) TrV rhombohedral crystal diffraction image (type I). The image corresponds to a 10 s exposure time and 0.3° oscillation angle with a crystal-to-detector distance of 300 mm. This image was obtained at the ESRF, Grenoble (beamline ID14-1, wavelength 0.93 Å), using a quantum ADSC Q4R CCD detector. (*b*) TrV orthorhombic crystal diffraction image (type II). The image corresponds to a 10 s exposure time and 0.25° oscillation angle with a crystal-to-detector distance of 270 mm. This image was obtained at the ESRF, Grenoble (beamline ID23-1, wavelength 0.95 Å). Spots are visible at the edge of the plate where the resolution is 2.5 Å. The diffraction pattern goes well beyond the water ring (this is not the case in pattern *a*).

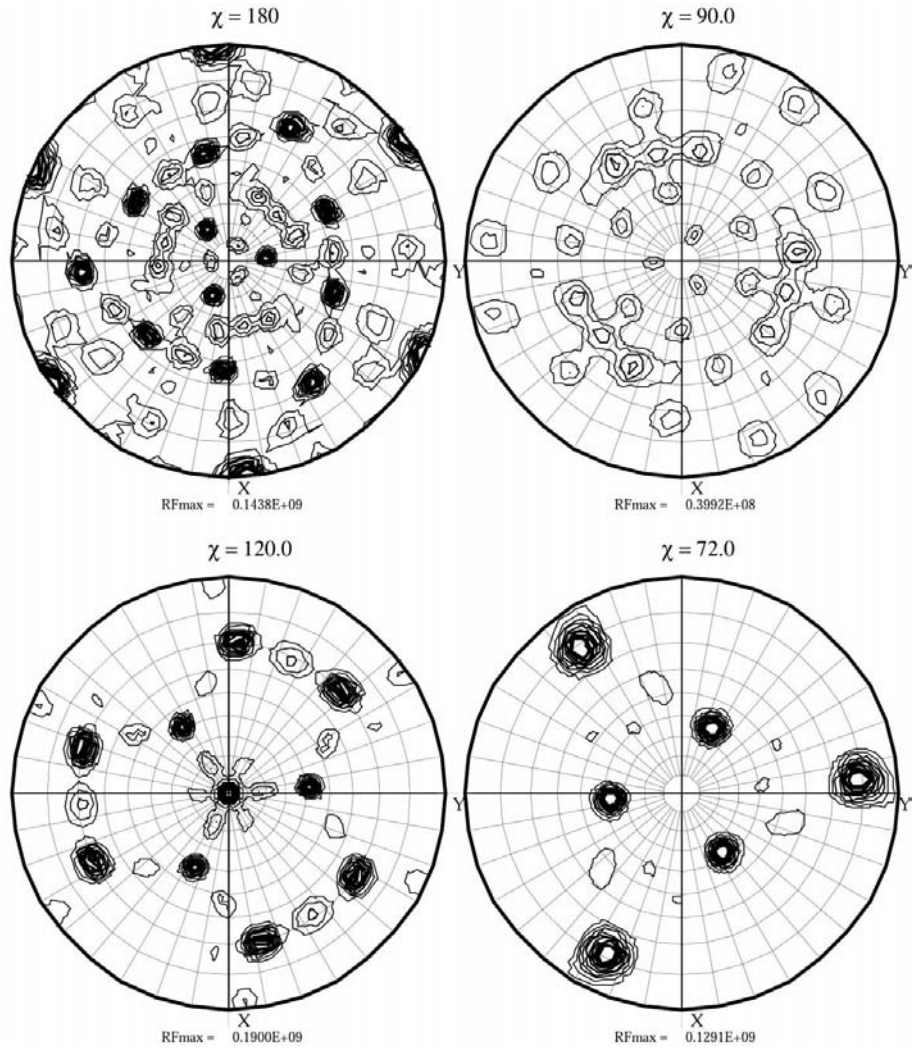

Figure 4
Self-rotation function output from *MOLREP* (see text).

the determination of the structure of the TrV particle to 3.2 Å. The availability of a complete 2.5 Å resolution data set in the near future will allow for the determination of a much more accurate atomic model and the investigation of the possible presence of metal ions and other factors stabilizing the capsid. This structure will also provide a wealth of information on the amino acids that are important for viral assembly and for the interaction with cellular receptors. Furthermore, an accurate comparison with the available structure of other viruses will be extremely useful, in combination with the genomic sequence, for an unambiguous classification of this insect virus. Finally, the availability of a 2.5 Å structure and the identification of the amino acids important for virulence will provide the basis for a rational approach to use TrV as a biological agent against the vector of *T. cruzi*.

This work is in memoriam to Dr O. A. Muscio who discovered TrV and has recently died. GSRD and DMAG are thankful to Dr J. La Torre (CEVAN) and to Dr A. M. Silva (Sequoia) who initiated this project, to Dr O. Fernández (CERZOS) for encouragement and support during the early stages of this project, and also to Dr N. Cazzaniga (DBBF–UNS) for helpful discussions. GS thanks S. Duqueroy for help during data collection and S. Bressanelli for assistance in data analysis. DMAG is a member of CONICET, Argentina. JL, JN and FAR are members of the CNRS, France. GS has a fellowship from the Ministère de l'Education Nationale et de la Recherche. JP acknowledges EMBO (ALTF 230/2002) and the European Commission (HPMF-CT-2002-01805) for fellowships. This work was partially supported by a UNS–PID grant to DMAG.

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