Phasing of the Triatoma virus diffraction data using a cryo-electron microscopy reconstruction

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Abstract

The blood-sucking reduviid bug *Triatoma infestans*, one of the most important vector of American human trypanosomiasis (Chagas disease) is infected by the Triatoma virus (TrV). TrV has been classified as a member of the Cripavirus genus (type cricket paralysis virus) in the Dicistroviridae family. This work presents the three-dimensional cryo-electron microscopy (cryo-EM) reconstruction of the TrV capsid at about 25 Å resolution and its use as a template for phasing the available crystallographic data by the molecular replacement method. The main structural differences between the cryo-EM reconstruction of TrV and other two viruses, one from the same family, the cricket paralysis virus (CrPV) and the human rhinovirus 16 from the Picornaviridae family are presented and discussed.

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Introduction

The Chagas disease is a human parasitic disease which occurs mainly in Central and South America (WHO-TDR, 2005). Its pathogenic agent is a flagellate protozoan named *Trypanosoma cruzi*, which is transmitted to humans and other mammals mostly by members of the subfamily Triatominae (family Reduviidae). Chagas disease occurs in two stages: the acute stage shortly after the infection, and the chronic stage that may develop over 10 years. The acute phase is usually asymptomatic, but may present symptoms of fever, lymphadenopathy, or a local skin nodule at the site of inoculation. The symptomatic chronic stage may affect the nervous system, digestive system and heart. Medication for Chagas disease is usually only effective when given during the acute stage of infection. If left untreated, Chagas disease can be fatal, in most cases due to the cardiomyopathy component (WHO-TDR, 2005). Triatoma virus (TrV) is a viral pathogen of *Triatoma infestans* (Muscio et al., 1987). TrV has initially been considered to be a member of the family Picornaviridae on the basis of physicochemical properties (Muscio et al., 1988). However, after the TrV genome was completely sequenced (Czibener et al., 2000), the virus was classified in a new viral family, Dicistroviridae (Mayo, 2002). This family contains a single genus, Cripavirus, whose type species is cricket paralysis virus (CrPV).

TrV virions consist of a non-enveloped capsid that encloses the viral genome, a single molecule of linear positive sense single-stranded RNA. The capsid encloses 60 repeats of four structural proteins VP1, VP2, VP3 and a minor one VP0 of 39 kDa, 37 kDa, 33 kDa and 45 kDa, respectively (Czibener et al., 2000), forming a T=1 (pseudo-triangulation p=3) icosahedron of about 30 nm diameter.

The goal of the present study was two-fold: to show the main large-scale structural differences between the TrV capsid...
surface obtained by cryo-EM and other two related virus, CrPV (belonging to the same new family, *Dicistroviridae*) and HRV16 (belonging to the *Picornaviridae* family), and to assess the performance of EM reconstructions as search models in the molecular replacement (MR) technique, for different levels of resolution, using the available crystallographic data (Rozas-Dennis et al., 2004).

We first studied TrV by transmission electron microscopy using negatively stained samples and performed a 3D reconstruction based on the obtained images. The high inhomogeneity of the samples and the featureless aspect of the particles limited the resolution of this reconstruction to about 35 Å. The lack of resolution overlap with the available crystallographic data impeded the successful use of this first reconstruction in the phasing process by molecular replacement. With similar samples but using cryo-electron microscopy (cryo-EM) we performed a three-dimensional reconstruction of TrV at better than 25 Å resolution which led to a solution of the MR problem. Here we describe the low resolution crystal structure of the TrV thus obtained, which provided the information necessary to start the phase extension process by non-crystallographic symmetry averaging. The phase extension has performed until 10 Å resolution and the map thus obtained is shown and compared to the cryo-EM reconstruction. The high resolution structure will be published in a forthcoming article (Squires, G., Pous., et al., 2008).

In the present case the TrV MR solution could also be obtained by using CrPV as a search model (21.8% identity and

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**Fig. 1.** Electron micrograph of TrV obtained using negative-stain transmission electron microscopy. TrV appear as smooth spherical particles. Note the presence of full viruses, empty capsids and other small non-identified particles. The scale bar represents 30 nm. High magnification of the three different kind of particles present in the preparation: full (A), empty (B) and small particles (C), respectively. The scale bar in C represents 10 nm.

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**Fig. 2.** Isosurface representation of the reconstruction based on negatively stained samples of TrV using Fourier coefficients up to 30 Å resolution. View along a 2-fold axis.

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**Fig. 3.** A slice of the reconstruction based on negatively stained samples of TrV.
36.8% similarity), but the use of an EM reconstruction based on the same particles which have been crystallized is a more robust method as sufficiently similar structures, useful for MR, are not always available.

**Electron microscopy and reconstructions**

Images of TrV obtained using negative-stain electron microscopy showed a non-homogeneous population of particles as shown in Fig. 1.

At least three different kinds of particles are seen: full spherical particles (Fig. 1A), empty capsids (Fig. 1B) and other smaller particles (Fig. 1C) whose origin we do not know yet. Both full and empty particles appear as smooth spheres of about 315 Å diameter. Some particles seem to be only partially emptied of their RNA genome.

A model-free 3D EM reconstruction was performed using these data and the *Rico* suite of programs based on symmetry adapted functions (Navaza, 2003, Estrozi and Navaza, in press).

Although we performed reconstruction trials (i.e. views assignment) using both full and empty particles separately, only the full particles provided consistent views through the view assignment iterations. The failure in reconstructing from the empty particles can be explained by a higher fragility of this kind of particles in comparison with the full ones. The empty particles would be then more affected (crushed) by the staining technique.

To perform the reconstruction, about 1000 particles were boxed from 4 different micrographs using the *X3D* program (Conway and Steven, 1999). They were Fourier–Bessel transformed within the 0 to 1/15 Å⁻¹ resolution range and angular frequencies (Bessel orders) up to \( \pi \times \frac{\text{Diameter}}{\text{Resolution}} \approx 70 \) were retained.

Second, for each image, the center was initially estimated by the SCA method (Estrozi et al., 2007) and each independent view, in 2.4 degrees steps, was assessed by comparison with the projection of the best icosahedral functions (in a least-squares sense) using angular frequencies up to 30 and radial samples up to 1/30 Å⁻¹. For each micrograph, the image with view parameters leading to the best-correlated reconstruction was taken as a reference and the view parameters of the remaining images were redetermined and refined taking this reference into account. At this point, only the full particles led to reference images whose assigned views were consistent i.e. they did not change if a different reference image was chosen. The center of each projection was also refined and a final cycle of view/center refinement was carried out at full resolution (15 Å). This last refinement step was repeated until convergence of the parameters, using data from the four micrographs simultaneously after contrast transfer function (CTF) correction. Most of the 1000 images were discarded due to the low correlation values presented. Only 120 particles have been retained to perform the final reconstruction. The zeros of the CTF were searched as described previously (Conway and Steven, 1999) and phase-flip corrections were calculated for each group of images (Erickson and Klug, 1971). Correction for the CTF amplitude decay was applied (Gaussian deconvolution (Conway and Steven, 1999)) although it did not improve the reconstruction.

The third and final step consisted in building the final reconstruction from the oriented and centered images. To minimize the introduction of noise in the reconstructions, data near the zeros of the CTF functions were eliminated. The reconstruction thus obtained is shown in Fig. 2. The isosurface representation of TrV displays five protuberances drawing a star on each of the icosahedral 5-fold axes, with a triangular plateau centered on each of the icosahedral 3-fold axis. Its resolution was estimated to be...
about 35 Å using Fourier Shell Correlation (FSC) with a threshold of 0.5. Looking at a slice of this reconstruction (Fig. 3) we observe the clear absence of internal structure due to the exclusively external coverage of the full particles by the stain.

Cryo-electron microscopy images of the same sample of TrV showed again a non-homogeneous viral particles population (Fig. 4). We distinguished also three different kind of particles: full particles (Fig. 4A), empty capsids (Fig. 4B) and small particles (Fig. 4C).

The same reconstruction procedure was applied to 1400 TrV cryo-EM images and in this case, and contrary to the negative-stained data, only the empty particles gave rise to reliable reconstructions. This fact could be explained by the disordered organization of the genetic material inside the full particles which would “interfere” with the featureless surface of the capsid during the view assignment step. The resolution range included in the calculations was between 0 to 1/8 Å⁻¹ and only Bessel orders below 128 were retained.

The final view and center refinement and reconstruction steps were carried out at full resolution (8 Å) after applying CTF correction in the same way as in the previous negative-stain reconstruction. 305 cryo-EM images were retained to produce the final 3D reconstruction, selected according to their correlations with the corresponding projections of the resulting reconstruction. The map, calculated using Fourier coefficients up to 15 Å resolution is shown in Figs. 5 and 6. However, resolution measures like Fourier Shell Correlation (FSC) (van Heel, 1987) and spectral signal-to-noise ratio (SSNR) (Unser et al., 1987) indicate that the reconstruction has 25 Å resolution according to the fall off of the plots in Figs. 7A and B. Interestingly, as shown in the next section, the 15 Å map has been successfully used to phase crystallographic data starting at 25 Å resolution. This might support the ideas presented in (van Heel and Schatz, 2005) about the criteria for cryo-EM resolution estimation.

The isosurface representation of TrV displays mainly the same features as those obtained with the negative-stain electron microscopy 3D reconstruction but, as expected, the cryo-EM reconstruction presents more well defined spikes around the 5-fold axis and internal information is now visible (Fig. 6).

**Use of the 3D EM reconstruction to solve the crystal structure**

In order to test the feasibility of phasing using exclusively our low resolution EM model, we used the TrV crystallographic...
data (type II crystals) reported by Rozas-Dennis et al. (2004) which was 98% complete in the 25–15 Å range, containing 5470 independent reflections therein. The AMoRe program (Navaza, 1994) allowed us to build a low resolution crystal structure using as the search model the TrV cryo-EM reconstruction. Data within the 25 to 15 Å range was needed in order to find a contrasted solution. This means that structural information is present beyond the limits proposed by the FSC and SSNR procedures.

In cryo-EM what is reconstructed is the perturbation in scattering intensity due to the virus particle relative to the background vitreous ice scattering intensity. In X-ray, diffraction data is usually collected at “high” resolution as the low resolution part of the spectrum contains solvent contribution which is only qualitatively explained by models and is not essential to recover the macromolecular atomic structures. An estimation of the solvent contribution to the structure factors may be done by invoking Babinet’s principle. In this approximation, the solvent contribution is taken into account by two parameters, a Gaussian resolution dampening factor, $B_{sol}$, and the ratio between the average solvent density and the average protein density, $K_{sol}$, through the expression

$$F_{\text{obs}}(h) = F_{\text{virus}}(h) \left(1 - K_{sol} \exp\left(-B_{sol} |h|^2 \right) \right).$$

Very often the optimized values of the bulk-solvent correction parameters have no physical meaning (Glykos and Kokkinidis, 2000). In our case, the best results were obtained using $K_{sol} = 1$ and $B_{sol} = 200$, although the solution was recognizable without bulk-solvent correction.

The orthorhombic (P2₁2₁2₁) unit cell has parameters $a = 336$, $b = 351$, $c = 332$ (Å), and the question is how many virus particles it contains. If they accommodate in a close-packed configuration they would occupy $\pi/\sqrt{18}$ of the available volume. With 2 particles per unit cell the particle diameter would be 303 Å, close to the experimentally observed value (with 3 particles, 264 Å; with 1 particle, 381 Å). Since there are 4 space group symmetry operations and only 2 virions within the unit cell, only half a virus is crystallographically independent. Therefore, the virus center of mass must occupy one of the two-fold site-symmetry positions.
which are, in fractional coordinates, (0, 0, z) or (0, 1/2). Although this information was not used in the molecular replacement problem, it allowed us to quickly assess the putative solutions. The oriented and translated model is presented in Fig. 8 together with some symmetry related particles to show the crystal packing. This molecular replacement solution was used to start the phase extension process known as non-crystallographic symmetry (NCS) averaging.

When using the reconstruction obtained with the negative-stained micrographs (Fig. 2) the correct position of the virus was not found despite the overall similarity with the cryo-EM reconstruction (Fig. 5). The reason of this failure probably stems from the small resolution overlap between the X-ray and the EM data.

Although similar results could have been obtained starting from atomic models, for instance the coordinates of CrPV, we wanted to show that a low resolution and noisy EM map may lead directly to an atomic structure without intervention of atomic models or experimental phases which are always difficult to obtain.

The NCS phase extension has been performed until 10 Å resolution and the map thus obtained is shown in Figs. 9 and 10. The FSC between this map and the cryo-EM reconstruction (Fig. 11) indicated an average correlation of 43% in the 25–
20 Å and of 29% in the 25–15 Å resolution range, supporting the argument that there is useful information beyond the limit indicated by the standard cryo-EM resolution measures. The oscillations observed in the FSC plot (Fig. 11) are probably due to the incompleteness of the X-ray data at low resolution.

Comparison of the 5-fold axis between TrV, CrPV and HRV16

Most of picornaviruses (members of Picornaviridae family) have a similar topography as previously described for poliovirus (PV) (Hogle et al., 1985) and human rhinovirus (HRV) (Rossmann et al., 1985, Colonno et al., 1996). These viruses exhibit a star-shaped 5-fold axis surrounded by a depression called “the canyon” and another (small) spike at the 3-fold axis as shown in Fig. 12B (small arrow). PV (Colston and Racaniello, 1994) and the major group of HRV (Rossmann et al., 1985, Colonno et al., 1988) hide their receptor site at the base of this “canyon” (Fig. 12B big arrow). However, some picornaviruses, as the aphthovirus and the cardiovirus, do not present this depression around each 5-fold axis and other sites act as binding sites for the receptors.

Based on its biophysical properties, TrV was initially classified as a member of the Picornaviridae family (Muscio et al., 1988) before the sequencing of its genome (Czbener et al., 2000). Thereafter TrV was reclassified in the Dicistroviridae family (Mayo, 2002).

Compared to a member of the Picornaviridae family (HRV16), the biggest structural difference observed between TrV and HRV16 is the absence of the “canyon” around each 5-fold axis as shown in Fig. 12B and F. This depression is also absent in the CrPV structure (another member of the Dicistroviridae family) as shown in Fig. 12D. In the TrV reconstruction, we observed some protuberances around each 5-fold axis. Additionally, the star-shaped 5-fold axis formed by VP1 (blue) jelly-roll visualized in the HRV16 structure is smaller in the CrPV ones and is completely absent in the TrV reconstruction. In fact a depression is found at this level, in contrast to most of the picornaviruses and to the CrPV. Fig. 12 shows the topography of HRV16, CrPV and TrV around the 5-fold axis where the colors blue, green and red code for VP1, VP2 and VP3, respectively. The TrV reconstruction has been colored according to the immediate CrPV protein, VP1, VP2 or VP3, after fitting into the TrV map (Fig. 13). The fitting was performed with the help of the URO program (Navaza et al., 2002). Besides the star-shaped 5-fold axis, long CrPV helices are standing out of the map around the two-fold axis, as seen in Fig. 13.

Conclusions

In this work we presented the three-dimensional cryo-EM reconstruction of the TrV capsid at about 25 Å. This reconstruction shows that, in contrast to the Picornaviridae family to which TrV was thought to belong to, the characteristic receptor binding site called “canyon” located at the surface of the viral capsid is absent. This suggests an alternative site and mechanism of receptor attachment like for the CrPV (Tate et al., 1999) but, unlike CrPV – the representative “type species” of the Cripavirus genus – TrV presents spikes around the 5-fold axis forming a “crown”, a peculiar feature of this member of the Dicistroviridae family recently created to accommodate many of the previously called “picorna-like virus” (Büchen-Osmond, 2006). Despite the facts that both picorna-like plant viruses and insect viruses share similar tertiary and quaternary structures with animal picornaviruses (Lin and Johnson, 2003, Tate et al., 1999, Squires, G., Pous, J., et al., 2008) and that in the majority of animal picornaviruses the canyon is associated to the binding of receptors consisting of a series of immunoglobulin superfAMILY domains (Rossmann et al., 2002) (and references therein), the missing canyon in both plant and insect capsid surfaces supports the conjecture that the canyon in picornaviruses is an adaptive response to the antibody-mediated defense of mammal host to virus infection, as was suggested from the CrPV structure analysis (Rossmann and Tao, 1999).

The main difficulty in improving the resolution of the presented reconstruction comes from the fact that the population of viral particles was not homogeneous. Indeed, most of the observed capsids were only partially filled by RNA. This made difficult to separate images of the full particles from the empty ones. In addition, the abundant presence of a third kind of smaller lips-shaped particles of 13.5 nm long and 7 nm wide which were sometimes superimposed to the viral capsids certainly contributed to limit the attained resolution. Up to now the nature of these objects remains unknown but we may expect of these particles to be a viral intermediate of TrV thus explaining the high proportion of empty (partially empty) particles in comparison to full viruses in our samples. Despite further efforts being made during the sample purification steps, the heterogeneity of the observed particles may suggest that modifications might be still happening after purification.

Nevertheless despite this limited resolution it was shown that the cryo-EM reconstruction of TrV contained enough information, quite beyond the value estimated by FSC and SSNR, as compared to that based on negatively stained samples, to solve
the crystal structure at the molecular replacement level; subsequent phase extension by non-crystallographic-symmetry averaging (beyond 10 Å) is on the way. The solution of the high resolution atomic structure of TrV will be published in a forthcoming article and the presented reconstructions are available at http://mem.ibs.fr/TrV.

Materials and methods

Preparation of TrV particles

TrV was purified from infected triatomines, essentially following the procedure described by Rozas-Dennis et al. (2002), with minor modifications. Whole bodies (adults and fifth-instar nymphs) of ten 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 mM NaCl, 1 mM MgCl2, 50 mM Tris–HCl pH 7.5) and chloroform. The homogenate was filtered and centrifuged at 4400 g for 20 min at 277 K. The supernatant solution was centrifuged at 35,000 g 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 116,000 gavl overnight at 277 K. The resulting virus band was collected with a peristaltic pump (Miniplus 3 Gilson) and dialysed against 500 mM KCl, 1 mM MgCl2 and 50 mM Tris–HCl pH 7.5. Micrographs were taken with a peristaltic pump (Miniplus 3 Gilson) and dialysed against 500 mM KCl, 1 mM MgCl2 and 50 mM Tris–HCl pH 7.5.

Electron microscopy

Triatoma virus at a concentration of 0.6 mg/ml of virus protein were first applied to the clean side of carbon on mica (carbon/mica interface) and negatively stained with 1% (w/v) ammonium molybdate. A grid was placed on top of the carbon film, which was subsequently air dried. Micrographs were taken with a CM12 microscope (FEI Eindhoven, The Netherlands) operating at 120 kV and a nominal magnification of 45,000× at 120 kV and a nominal magnification of 45,000×. The resulting virus band was collected with a peristaltic pump (Miniplus 3 Gilson) and dialysed against 500 mM KCl, 1 mM MgCl2 and 50 mM Tris–HCl pH 7.5.

The isosurface representations of the reconstructed densities were rendered by using the programs *O* (Jones, 1978) and *Py- mol* (DeLano, 2002).

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