Coxsackievirus B3 protease 3C: expression, purification, crystallization and preliminary structural insights

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Viral proteases are proteolytic enzymes that orchestrate the assembly of viral components during the viral life cycle and proliferation. Here, the expression, purification, crystallization and preliminary X-ray diffraction analysis are presented of protease 3C, the main protease of an emerging enterovirus, coxsackievirus B3, that is responsible for many cases of viral myocarditis. Polycrystalline protein precipitates suitable for X-ray powder diffraction (XRPD) measurements were produced in the presence of 22–28% (w/v) PEG 4000, 0.1 M Tris–HCl, 0.2 M MgCl₂ in a pH range from 7.0 to 8.5. A polymorph of monoclinic symmetry (space group C2, unit-cell parameters $a = 77.9$, $b = 65.7$, $c = 40.6$ Å, $\beta = 115.9^\circ$) was identified *via* XRPD. These results are the first step towards the complete structural determination of the molecule *via* XRPD and a parallel demonstration of the accuracy of the method.

1. Introduction

Enteroviruses (EVs) are pathogens of significant importance that infect 50 million people each year in the US and possibly more than a billion worldwide (Morens & Pallansch, 1995; Pallansch & Roos, 2001). These viruses are responsible for a variety of diseases from asymptomatic or mild febrile and respiratory illnesses to potentially fatal diseases (Yin-Murphy & Almond, 1996). For instance, poliomyelitis in infants and children is the most significant disease caused by poliovirus, an enterovirus which invades the nervous system (Huang & Shih, 2015). Other non-polio enteroviruses are the most common causes of aseptic meningitis, pericarditis, myocarditis and respiratory infections (Racaniello, 2007).

Enteroviruses and consequently coxsackieviruses are members of the *Picornaviridae* family (Romero & Modlin, 2015). All picornaviruses recognize specific receptors on the surface of the host cells, which they bind as an initial step in their entry into it (Rossmann *et al.*, 2000). The mechanism of entry involves binding to the coxsackievirus and adenovirus receptor (CAR), a transmembrane protein of epithelial tight junctions (Cohen *et al.*, 2001). Further binding to the decay-accelerating factor (DAF), which acts as a co-receptor, is required for endocytosis of the virus particles (Coyne & Bergelson, 2006). Binding to CAR and DAF activates the

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CAR-associated tyrosine kinase Fyn, which further facilitates the activation of signalling pathways that lead to caveolae-mediated endocytosis (Coyne & Bergelson, 2006). These interactions are also required for the infection of other tissues mentioned above (Epelman *et al.*, 2015).

The group of coxsackieviruses, which belongs to this genus, contains several serotypes which are subgrouped into coxsackieviruses A (CAVs) and coxsackieviruses B (CBVs) (Santti *et al.*, 2000). Coxsackievirus B enters the human body through the intestinal epithelium (Grove & Marsh, 2011), but also attacks other types of organs such as lymphoid (Matteucci *et al.*, 1985), myocardial (Lodge *et al.*, 1987) and neural tissues (Rhoades *et al.*, 2011). Coxsackieviruses can cause acute clinical phenomena ranging from mild febrile illness to more severe diseases, including viral myocarditis (Gebhard *et al.*, 1998), aseptic meningitis and encephalitis (Feuer *et al.*, 2003), pancreatitis (Huber & Ramsingh, 2004) and possibly type 1 diabetes (Drescher *et al.*, 2004).

The genome of enteroviruses consists of a single-stranded positive-sense RNA (Romero & Modlin, 2015). After being delivered into the host cell, viral RNA is translated *via* the host cell machinery into a polyprotein precursor that is cleaved by two chymotrypsin-like viral cysteine proteases (Malcolm, 1995; Seipelt *et al.*, 1999), protease 2A (2A^{pro}) and protease 3C (3C^{pro}), in order to produce mature viral proteins (Weidman *et al.*, 2003). 3C^{pro}, a proteolytic enzyme encoded by various genera of the *Picornaviridae* family (Malcolm, 1995), is responsible for the majority of the viral polyprotein maturation cleavages and is therefore central to virus replication (Porter, 1993). Autocatalytic cleavage of a precursor 3CD protein yields mature 3C^{pro} and 3D^{pol} (Burns *et al.*, 1989; Probst *et al.*, 1998). 3D^{pol} is the active RNA-dependent RNA polymerase (Harris *et al.*, 1992). 3C^{pro} is also responsible for the proteolytic cleavage of a large number of transcription factors in the host cell, therefore inhibiting the action of cellular RNA polymerases I, II and III (Lin *et al.*, 2009). These factors include the TATA-binding protein (TBP), which affects Pol II transcription (Clark *et al.*, 1993), the CREB transcription factor (Yalamanchili, Datta *et al.*, 1997), the octamer-binding protein (Oct-1; Yalamanchili, Weidman *et al.*, 1997b) and the transcription activator p53 (Weidman *et al.*, 2001).

Owing to the significant role of 3C^{pro} in the viral life cycle, this molecule has been considered as a possible antiviral target (Wang & Liang, 2010). Several crystallographic studies have been performed on viral 3C proteases (Costenaro *et al.*, 2011; Cui *et al.*, 2011; Lu *et al.*, 2011; Tan *et al.*, 2013). Although 3C proteases have an active-site cysteine nucleophile, their fold is similar to that of chymotrypsin-like serine proteases (Lall *et al.*, 2004). Previous studies of coxsackievirus B3 (CVB3) protease 3C have shown that crystals obtained from this molecule have monoclinic symmetry (Tan *et al.*, unpublished work; Lee *et al.*, 2009). However, corresponding structural data in the Protein Data Bank are limited, and a wider range of crystallization conditions such as pH, precipitants and the presence of ligands acting as possible inhibitors needs to be explored.

Information on designing inhibitors that bind in the protease active site can be obtained *via* crystallographic studies (Lall *et al.*, 2004). The three-dimensional structures of proteins, which are obtained by X-ray crystallography, provide valuable information about the surface of the protein, which can then be targeted by a specific ligand with a complementary surface (Blundell *et al.*, 2002). As a next step, co-crystallization of the protein with selected ligands is performed in order to identify ligand-binding sites and ensure that they match the predicted sites (Carvalho *et al.*, 2010). Several antiviral drugs such as oseltamivir (Tamiflu), which is used to treat influenza, and nelfinavir (Viracept), which is used in the treatment of HIV, have been designed through a structure-based approach (Menéndez-Arias & Gago, 2013) using the three-dimensional structures of influenza virus neuraminidase (Kim *et al.*, 1999) and HIV-1 protease (Wlodawer & Vondrasek, 1998), respectively.

In this study, we present the expression, purification, crystallization and X-ray powder diffraction (XRPD) preliminary data analysis of the 3C^{pro} protein from CVB3. The application of powder diffraction to proteins is an efficient method for high-throughput crystal screening and polymorph identification (Norrman *et al.*, 2006; Collings *et al.*, 2010; Papageorgiou *et al.*, 2010; Karavassili *et al.*, 2012; Valmas *et al.*, 2015; Fili *et al.*, 2015), as well as structure solution (Margiolaki & Wright, 2008; Karavassili & Margiolaki, 2016) and refinement (Margiolaki *et al.*, 2013).

2. Materials and methods

2.1. Protease 3C production

Plasmid pET-24a(+) was used as a vector for protein production. The sequence encoding protease 3C from CVB3 was synthesized (GenScript) and cloned into pET-24a(+) by restriction/ligation, with a hexahistidine-coding sequence at the 3' end. T7 Express IqplysS competent *Escherichia coli* cells were transformed by the plasmid. A single colony was picked to inoculate a 5 ml preculture in Terrific Broth medium containing 50 mg ml⁻¹ kanamycin and 35 mg ml⁻¹ chloramphenicol. After overnight incubation at 37°C and 200 rev min⁻¹, the preculture was used to inoculate 100 ml Terrific Broth medium containing 50 mg ml⁻¹ kanamycin and 35 mg ml⁻¹ chloramphenicol. When an optical absorption of 0.6 (at a wavelength of 600 nm) was reached, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce overexpression. The culture was then left overnight at 23°C and centrifuged for 10 min (4000 rev min⁻¹). The resulting pellet was resuspended in 2 ml lysis buffer consisting of 50 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole, 5% glycerol, 0.1% Triton-X. The cell suspension was lysed by one cycle of freezing (−80°C) and thawing (+10–15°C), the addition of 2 ml lysis buffer and lysozyme (0.15 mg ml⁻¹) and sonication (Sonics Vibra-Cell VCX 130 PB sonicator). After cell lysis, lysates were separated by centrifugation at 4°C (10 000 rev min⁻¹) twice for 30 min each time. The supernatant containing the soluble fraction of 3C^{pro} was then

Table 1
Protease 3C production information.

Source virus	Coxsackievirus B3 (strain Nancy)
Expression vector	pET-24a(+)
Expression host	T7 Express IqpLysS competent <i>E. coli</i> strain
No. of residues	184
Complete amino-acid sequence of the construct produced	MGPAFEFAVAMMKRNSSTVKTEYGFTMLGIYDR-WAVLPRHAKPGPTILMNDQEVGVLDKELVDK-DGTNLELTLLKLNREKFRDIRGFLAKEEVEV-NEAVLAINTSKFPNMYIPVGQVTEYGFLNLGG-TPTKRLMYNFPTRAGQCGVLMSTGKVLGIH-VGGNGHQGFSAAALLKHYFNDEQLEHHHHHH

analyzed by 15% SDS-PAGE gel electrophoresis (OmniPAGE Mini System; Fig. 1). Macromolecule-production information is summarized in Table 1.

Purification was performed in an ÄKTA Start system (GE Healthcare) by immobilized metal-affinity chromatography (IMAC) on a 1 ml HisTrap HP column (GE Healthcare). A buffer consisting of 300 mM NaCl, 50 mM Tris-HCl pH 7.5 was used as the binding buffer. The column was then washed using 15 column volumes (CV) of washing buffer (300 mM NaCl, 50 mM Tris-HCl, 30 mM imidazole pH 7.7). Elution of the protein was performed in 7 CV of elution buffer (300 mM NaCl, 50 mM Tris-HCl, 500 mM imidazole pH 7.77). After the IMAC purification, the fractions were analysed on a Coomassie Blue-stained 15% SDS-PAGE gel and those containing the target protein were pooled together and loaded onto a HiLoad Superdex 200 16/60 (GE Healthcare) size-exclusion chromatography column equilibrated with 10 mM HEPES, 300 mM NaCl pH 7.5 (Fig. 1). After elution, the

Table 2
Crystallization.

Method	Batch crystallization (salting out)
Container	Eppendorf microcentrifuge tubes (1.5 ml)
Temperature (K)	298
Protein concentration (mg ml ⁻¹)	10
Buffer composition of protein solution	10 mM HEPES, 300 mM NaCl pH 7.5
Composition of precipitant solution (initial condition)	0.1 M Tris-HCl pH 8.5, 0.2 M MgCl ₂ , 22% PEG 4000

targeted fractions were collected and the protein was concentrated to 10 mg ml⁻¹ (Amicon Ultra-4 centrifugal filter unit), flash-frozen in liquid nitrogen and stored at -80°C.

2.2. Crystallization

A starting crystallization condition (0.1 M Tris-HCl pH 8.5, 0.2 M magnesium chloride, 22% PEG 4000), suitable for single-crystal production *via* the sitting-drop method, was originally selected from commercial screens. Although useful as a starting point, the condition mentioned above needed further exploration around the initial pH value (7.00–8.50) and PEG 4000 concentration (22–28%) in order to be optimal for polycrystalline precipitate production. Following this process, 16 different crystallization buffers were produced with variations in pH and PEG 4000 concentration (Supplementary Table S1). Crystallization was performed using the salting-out method (Hofmeister, 1888) in batch. 50 µl protein solution (10 mg ml⁻¹) was placed in 16 × 200 µl tubes and mixed with equal amounts of the previously produced crystallization buffers. The samples were crystallized at 298 K. After 48 h, the solution yielded ~10 µl of polycrystalline material (Fig. 2) at the bottom of each tube. The average size of a single crystallite (~0.1–1 mm) in such precipitates is usually far too small for conventional single-crystal diffraction, but is ideal for X-ray powder diffraction (XRPD). Crystallization information is summarized in Table 2.

Initially, XRPD profiles were collected for each of the 16 samples (see §2.3). However, the data quality underlined the necessity for further improvement of the crystallization conditions. Therefore, crystallization buffers containing greater than 26% PEG 4000 and/or with a pH value higher than 8.0 were rejected owing to observed limited data resolution. In addition, larger sample volumes were necessary for multiple XRPD measurements in order to assess the reproducibility of the results. For this reason, four samples were prepared by mixing 350 µl protein solution (10 mg ml⁻¹) with equal amounts of crystallization buffers (buffers A–D in Supplementary Table S1) in 1.5 ml tubes. After 72 h, each sample yielded ~100 µl of polycrystalline material (Fig. 2), an amount sufficient for additional XRPD measurements.

2.3. Data collection and processing

Data collection was performed using different instruments and sources in order to optimize data quality. As radiation damage can be a serious obstacle to the collection of high-quality data, a large quantity of protein sample in conjunction

Figure 1
Analysis of the expression and purification of CVB3 3C^{pro}. (a) Analysis of 3C protease expression in *E. coli* on a Coomassie Blue-stained SDS-PAGE gel. Lane 1, sample from the supernatant after overnight expression. Lane M, molecular-weight marker (labelled in kDa). (b) Analysis of the purification of CVB3 3C^{pro} expressed in *E. coli* on a Coomassie Blue-stained SDS-PAGE gel. Lane M, molecular-weight marker (labelled in kDa); lanes marked with an asterisk are the selected fractions containing CVB3 3C^{pro} after size-exclusion chromatography.

with a wise strategy can reinforce the quality of the measurements with respect to angular resolution (FWHM) and d -spacing resolution. Profiles were obtained at the European Synchrotron Radiation Facility (ESRF), France and by using laboratory instrumentation (PANalytical X'Pert PRO). XRPD data were collected at room temperature (RT) on the High Resolution Powder Diffraction Beamline ID31 (currently ID22) at ESRF (Fitch, 2004) [$\lambda = 1.29983$ (1) and 1.30008 (5) Å] in order to achieve high angular resolution associated with reduced peak overlap and signal-to-noise ratio. Additional data were collected in our laboratory using a PANalytical X'Pert PRO diffractometer in order to achieve enhanced d -spacing resolution owing to reduced radiation damage ($\lambda = 1.541874$ Å).

All samples were loaded into borosilicate glass capillaries of 1 mm diameter. The capillaries were then centrifuged in order to enhance crystal packing. The excess mother liquor was removed and the capillaries were sealed with grease to prevent dehydration. The capillaries were mounted on the diffract-

ometer and spun at $1000 \text{ rev min}^{-1}$ to ensure adequate powder averaging.

For the synchrotron XRPD data collection, radiation-damage effects owing to the intense synchrotron beam, such as considerable changes in the unit-cell parameters along with gradual peak broadening, were limited by capillary translation. Approximately ten scans were collected per sample, translating every 2 min, at room temperature. Appreciable changes were observed in the data after several patterns had been collected. In order to increase counting statistics without compromising data quality, identical scans collected from fresh parts of the samples were summed together. In the case of data collection using the laboratory diffractometer, no radiation damage was observed even after 24 h of measurements.

All patterns were typically indexed using the *DASH* (Boultif & Louër, 1991; David *et al.*, 2006) or *HighScore Plus* software (Degen *et al.*, 2014), employing the fitted positions of at least the first 20 reflections of the synchrotron profiles. From

Figure 2
Polycrystalline samples of CVB3 3C^{pro}. The images correspond to samples with codes 3C_2 (upper left), 3C_9 (upper right), 3C_14 (lower left) and 3C_D (lower right). All of the crystals adopt the same symmetry (monoclinic).

Table 3
Data collection and processing.

Diffraction source	ID31, ESRF
Wavelengths (Å)	1.29983 (1), 1.30008 (5)
Temperature (K)	293
Detector	APD†
Exposure time per scan (s)	120
Space group	<i>C2</i>
<i>a</i> , <i>b</i> , <i>c</i> (Å)	78.073 (1), 65.577 (6), 40.497 (1)
α , β , γ (°)	90, 115.415 (1), 90
Resolution range (Å)	48.1–7.0
Total No. of reflections	4277

† Nine Si(111) analyser crystals each followed by a point detector [avalanche photodiode (APD) detectors].

the extracted data, we were able to determine the symmetry and unit-cell parameters for all samples. In order to obtain accurate values of the unit-cell parameters and to characterize the peak shape and background coefficients without a structural model, Pawley fits (Pawley, 1981) were performed using *PRODD* (Wright, 2004) as well as *HighScore Plus*. Data-collection and processing statistics are summarized in Table 3.

3. Results and discussion

The presence of 3C^{pro} in the soluble fraction of the bacterial lysate was checked using Coomassie Blue-stained SDS–PAGE gels (Fig. 1*a*). The fractions obtained after the various purification steps were also analyzed using Coomassie Blue-stained SDS–PAGE gels for further validation of the procedure (Fig. 1*b*). Batch crystallization using concentrated protein solution (10 mg ml^{−1}) was successful.

The first series of samples (codes 3C₁–16 in Supplementary Table S1) gave diffraction patterns of up to 10.6 Å resolution, indicating low sample crystallinity. Optimization of the crystallization conditions was carried out in order to improve the crystal quality, and thus four new samples were produced (codes 3C_A–3C_D in Supplementary Table S1). Improved

data quality was achieved and the *d*-spacing resolution was increased to 7.0 Å, which was sufficient for the accurate determination of space group and unit-cell parameters.

The XRPD data indicated no structural modifications or alterations in the diffraction patterns (peak positions) throughout the crystallization conditions studied, as illustrated in Fig. 3. Indexing indicated that all samples (prepared in the pH range 7.0–8.5 and 22–28% PEG 4000) contained crystals with monoclinic symmetry (space group *C2*). A subsequent Pawley analysis of all data sets, performed using *PRODD* and verified *via HighScore Plus*, was satisfactory for all profiles (Figs. 4 and 5), with typical agreement factors of $R_{wp} = 12.745\%$ and $\chi^2 = 1.6506$ for the sample crystallized at pH 8.00 and 26% PEG 4000. At the end of the refinement process, accurate unit-cell parameters were extracted and are listed in Supplementary Table S1 [$a = 78.073$ (1), $b = 65.577$ (6), $c = 40.497$ (1) Å, $\beta = 115.415$ (1)°]. According to Matthews coefficient calculation (Matthews, 1968; Kantardjieff & Rupp, 2003), in all different crystallization conditions the asymmetric unit contains one molecule, while the unit cell consists of four molecules with 46.26% solvent content (Matthews coefficient = 2.29 Å³ Da^{−1}). These observations are in good agreement with the previously reported structural model (PDB entry 3zyd; space group *C2*, unit-cell parameters $a = 76.85$, $b = 64.38$, $c = 39.36$ Å, $\beta = 116.41$ °; J. Tan, K. Anand, J. R. Mesters & R. Hilgenfeld, unpublished work).

Powder diffraction is an ideal technique for accurately recording reflections in the low 2θ region ($d > 30$ – 40 Å) and data usually extend to medium resolution (3–6 Å). The low-angle part of the diffraction profile is generally collected with a very high degree of accuracy and in a rather fast manner, since this part contains the strongest reflections. Also, it is in the low-angle part of the data set where the problem of overlapping reflections is least severe since the density of reflections increases with the cube of the distance from the reciprocal-space origin (Altomare *et al.*, 1995; David, 2004).

Figure 3
Surface plot of XRPD data from CVB3 3C^{pro} polycrystalline precipitates. Samples were crystallized in the pH range from 7 to 8.5 and PEG 4000 concentration range from 22 to 28%. Data were collected on ID22 at the ESRF [$\lambda = 1.29983$ (1) Å, room temperature]. No alteration in the diffraction peak positions is observed.

However, the high solvent contribution to the low-angle reflections necessitates particular attention in order to perform structure refinements and compute correlation coefficients between experimental and theoretical (based on a structural model) structure factors. Once a satisfactory description of the peak profiles is established, refinement of the structure can proceed. Isotropic temperature factors are employed for the description of the thermal motion of all atoms ($U_{\text{iso}} = B_{\text{iso}}/8\pi^2$). A Babinet's principle modification of all the atom scattering factors according to

$$f = f_0 - A_s \exp(-8\pi^2 B_s \sin^2 \theta / \lambda^2) \quad (1)$$

is employed in order to account for solvent scattering and to facilitate fitting the lowest angle part of the powder diffraction data (Margiolaki *et al.*, 2005; Basso *et al.*, 2005). Structure refinements are always performed *via* the combination of multiple data sets (Von Dreele, 2007; Margiolaki *et al.*, 2007; Margiolaki & Wright, 2008; Karavassili & Margiolaki, 2016), a combined Rietveld (Rietveld, 1969) and stereochemical restraint least-squares refinement approach (Von Dreele *et al.*, 2000; Von Dreele, 2001, 2005) and, more recently, the flexible rigid-body approach (Margiolaki *et al.*, 2013). The data presented in this article contain sufficient information for accurately identifying the space group and unit-cell parameters and for extracting intensities from the collected data sets using the Pawley method. Additional data with enhanced resolution are necessary to proceed with structure refinement

and further crystallization and diffraction experiments are currently ongoing.

Although XRPD provides medium-resolution data (3–10 Å), low-quality microcrystals can be studied, polymorph screening is a routine practice and time-resolved studies are also possible using this method. In addition, several recent studies have underlined the efficiency of the method for high-throughput polymorph screening in a range of crystallization conditions in order to extract both the structural characteristics of the protein and, more importantly, the binding affinity of selected small molecules (drug candidates; Karavassili *et al.*, 2012; Beckers *et al.*, 2015; Fili *et al.*, 2015; Valmas *et al.*, 2015; Karavassili & Margiolaki, 2016). In this study, we show that the CVB3 3C^{pro}, an important antiviral target, is amenable to XRPD, providing opportunities to determine the structures of target–inhibitor complexes in a wider range of conditions compared with single-crystal diffraction.

Our long-term aim is to further promote the application of the XRPD method as a powerful complementary tool in protein crystallography. Growing a single crystal is often time-consuming and success is never guaranteed; it is the major bottleneck in single-crystal diffraction. During the last decade, the use of powder data with proteins has improved from an era where useful crystallographic information could only be collected from single crystals with minimum sizes of 5–10 µm (Evans *et al.*, 2011) to modern times where high-quality data are routinely recorded and protein structures can be obtained from crystals of sizes of less than 0.1 µm (Von Dreele, 2003; Margiolaki *et al.*, 2005; Margiolaki & Wright, 2008). The

Figure 4

Pawley fit of the laboratory data set for CVB3 3C^{pro} (sample code 3C_D, pH 8.00, 26% PEG 4000; space group C2). The data were collected with a wavelength of 1.541874 Å using the X'Pert PRO diffractometer. The black, red and lower blue lines represent the experimental data, the calculated pattern and the difference between the experimental and calculated profiles, respectively. The vertical bars correspond to Bragg reflections compatible with space group C2. The refined background has been subtracted from the measured profile. The extracted unit-cell parameters are $a = 77.895$ (7), $b = 65.386$ (3), $c = 40.406$ (3) Å, $\beta = 115.3901$ (8)°, with agreement factors of $R_{\text{wp}} = 0.44\%$ and $\chi^2 = 3.282$. The total number of extracted intensities is 4613. The data are available as Supporting Information.

Figure 5

Pawley fit of the synchrotron data set of CVB3 3C^{pro} (sample code 3C_D, pH 8.00, 26% PEG 4000; space group C2). The data were collected on ID31 with a wavelength of 1.30008 (5) Å (room temperature). The black, red and blue lines represent the experimental data, the calculated pattern and the difference between the experimental and calculated profiles, respectively. The vertical bars correspond to Bragg reflections compatible with space group C2. The extracted unit-cell parameters are $a = 78.073$ (1), $b = 65.577$ (6), $c = 40.497$ (1) Å, $\beta = 115.415$ (1)°, with agreement factors of $R_{\text{wp}} = 12.745\%$ and $\chi^2 = 1.6506$. The total number of extracted intensities is 4277. The data are available as Supporting Information.

method can be combined with emerging techniques on nano/microcrystals such as electron diffraction (Shi *et al.*, 2013; Nannenga & Gonen, 2016) and serial femtosecond crystallography (Aquila *et al.*, 2012; Hirata *et al.*, 2014). The coming years will hopefully bring further methodological advances providing insight into important biological problems.

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References

- Altomare, A., Cascarano, G., Giacovazzo, C., Guagliardi, A., Moliterni, A. G. G., Burla, M. C. & Polidori, G. (1995). *J. Appl. Cryst.* **28**, 738–744.
- Aquila, A. *et al.* (2012). *Opt. Express*, **20**, 2706–2716.
- Basso, S., Fitch, A. N., Fox, G. C., Margiolaki, I. & Wright, J. P. (2005). *Acta Cryst.* **D61**, 1612–1625.
- Beckers, D., Degen, T., Nénert, G., Saslis, S., Logotheti, S., Karavassili, F., Valmas, A. & Margiolaki, I. (2015). *Acta Cryst.* **A71**, s510.
- Blundell, T. L., Jhoti, H. & Abell, C. (2002). *Nature Rev. Drug. Discov.* **1**, 45–54.
- Boulton, A. & Louër, D. (1991). *J. Appl. Cryst.* **24**, 987–993.
- Burns, C. C., Lawson, M. A., Semler, B. L. & Ehrenfeld, E. (1989). *J. Virol.* **63**, 4866–4874.
- Carvalho, A. L., Trincão, J. & Romão, M. J. (2010). *Ligand–Macromolecular Interactions in Drug Discovery: Methods and Protocols*, edited by A. C. Roque, pp. 31–56. Totowa: Humana Press.
- Clark, M. E., Lieberman, P. M., Berk, A. J. & Dasgupta, A. (1993). *Mol. Cell. Biol.* **13**, 1232–1237.
- Cohen, C. J., Shieh, J. T. C., Pickles, R. J., Okegawa, T., Hsieh, J.-T. & Bergelson, J. M. (2001). *Proc. Natl Acad. Sci. USA*, **98**, 15191–15196.
- Collings, I., Watier, Y., Giffard, M., Dagogo, S., Kahn, R., Bonneté, F., Wright, J. P., Fitch, A. N. & Margiolaki, I. (2010). *Acta Cryst.* **D66**, 539–548.
- Costenaro, L., Kaczmarek, Z., Arnan, C., Janowski, R., Coutard, B., Sola, M., Gorbalenya, E., Norder, H., Canard, B. & Coll, M. (2011). *J. Virol.* **85**, 10764–10773.
- Coyne, C. B. & Bergelson, J. M. (2006). *Cell*, **124**, 119–131.
- Cui, S., Wang, J., Fan, T., Qin, B., Guo, L., Lei, X., Wang, J., Wang, M. & Jin, Q. (2011). *J. Mol. Biol.* **408**, 449–461.
- David, W. I. F. (2004). *J. Appl. Cryst.* **37**, 621–628.
- David, W. I. F., Shankland, K., van de Streek, J., Pidcock, E., Motherwell, W. D. S. & Cole, J. C. (2006). *J. Appl. Cryst.* **39**, 910–915.
- Degen, T., Sadki, M., Bron, E., König, U. & Nénert, G. (2014). *Powder Diff.* **29**, S13–S18.
- Drescher, K. M., Kono, K., Bopegamage, S., Carson, S. D. & Tracy, S. (2004). *Virology*, **329**, 381–394.
- Epelman, S., Liu, P. P. & Mann, D. L. (2015). *Nature Rev. Immunol.* **15**, 117–129.
- Evans, G., Axford, D., Waterman, D. & Owen, R. L. (2011). *Crystallogr. Rev.* **17**, 105–142.
- Feuer, R., Mena, I., Pagarigan, R. R., Harkins, S., Hassett, D. E. & Whitton, J. L. (2003). *Am. J. Pathol.* **163**, 1379–1393.
- Fili, S., Valmas, A., Norrman, M., Schluckebier, G., Beckers, D., Degen, T., Wright, J., Fitch, A., Gozzo, F., Giannopoulou, A. E., Karavassili, F. & Margiolaki, I. (2015). *IUCrJ*, **2**, 534–544.
- Fitch, A. (2004). *J. Res. Natl Inst. Stand. Technol.* **109**, 133.
- Gebhard, J. R., Perry, C. M., Harkins, S., Lane, T., Mena, I., Asensio, V. C., Campbell, I. L. & Whitton, J. L. (1998). *Am. J. Pathol.* **153**, 417–428.
- Grove, J. & Marsh, M. (2011). *J. Cell Biol.* **195**, 1071–1082.
- Harris, K. S., Reddigari, S. R., Nicklin, M. J., Hämmerle, T. & Wimmer, E. (1992). *J. Virol.* **66**, 7481–7489.
- Hirata, K. *et al.* (2014). *Nature Methods*, **11**, 734–736.
- Hofmeister, F. (1888). *Arch. Exp. Pathol. Pharmacol.* **24**, 247–260.
- Huang, H.-I. & Shih, S.-R. (2015). *Viruses*, **7**, 6051–6066.
- Huber, S. & Ramsingh, A. I. (2004). *Viral Immunol.* **17**, 358–369.
- Kantardjieff, K. A. & Rupp, B. (2003). *Protein Sci.* **12**, 1865–1871.
- Karavassili, F. & Margiolaki, I. (2016). *Protein Pept. Lett.* **23**, 232–241.
- Karavassili, F., Giannopoulou, A. E., Kotsiliti, E., Knight, L., Norrman, M., Schluckebier, G., Drube, L., Fitch, A. N., Wright, J. P. & Margiolaki, I. (2012). *Acta Cryst.* **D68**, 1632–1641.
- Kim, C. U., Chen, X. & Mendel, D. B. (1999). *Antiviral Chem. Chemother.* **10**, 141–154.
- Lall, M. S., Jain, R. P. & Vederas, J. C. (2004). *Curr. Top. Med. Chem.* **4**, 1239–1253.
- Lee, C.-C., Kuo, C.-J., Ko, T.-P., Hsu, M.-F., Tsui, Y.-C., Chang, S.-C., Yang, S., Chen, S.-J., Chen, H.-C., Hsu, M.-C., Shih, S.-R., Liang, P.-H. & Wang, A. H.-J. (2009). *J. Biol. Chem.* **284**, 7646–7655.
- Lin, J.-Y., Chen, T.-C., Weng, K.-F., Chang, S.-C., Chen, L.-L. & Shih, S.-R. (2009). *J. Biomed. Sci.* **16**, 103.
- Lodge, P. A., Herzum, M., Olszewski, J. & Huber, S. A. (1987). *Am. J. Pathol.* **128**, 455–463.
- Lu, G., Qi, J., Chen, Z., Xu, X., Gao, F., Lin, D., Qian, W., Liu, H., Jiang, H., Yan, J. & Gao, G. F. (2011). *J. Virol.* **85**, 10319–10331.
- Malcolm, B. A. (1995). *Protein Sci.* **4**, 1439–1445.
- Margiolaki, I., Giannopoulou, A. E., Wright, J. P., Knight, L., Norrman, M., Schluckebier, G., Fitch, A. N. & Von Dreele, R. B. (2013). *Acta Cryst.* **D69**, 978–990.
- Margiolaki, I. & Wright, J. P. (2008). *Acta Cryst.* **A64**, 169–180.
- Margiolaki, I., Wright, J. P., Fitch, A. N., Fox, G. C. & Von Dreele, R. B. (2005). *Acta Cryst.* **D61**, 423–432.
- Margiolaki, I., Wright, J. P., Wilmanns, M., Fitch, A. N. & Pinotsis, N. (2007). *J. Am. Chem. Soc.*, **129**, 11865–11871.
- Matteucci, D., Paglianti, M., Giangregorio, A. M., Capobianchi, M. R., Dianzani, F. & Bendinelli, M. (1985). *J. Virol.* **56**, 651–654.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- Menéndez-Arias, L. & Gago, F. (2013). *Structure and Physics of Viruses: An Integrated Textbook*, edited by G. M. Mateu, pp. 599–630. Dordrecht: Springer.
- Morens, D. M. & Pallansch, M. A. (1995). *Human Enterovirus Infections*, edited by H. A. Rotbart, pp. 3–23. Washington: ASM Press.

- Nannenga, B. L. & Gonen, T. (2016). *Curr. Opin. Struct. Biol.* **40**, 128–135.
- Norrmann, M., Ståhl, K., Schluckebier, G. & Al-Karadaghi, S. (2006). *J. Appl. Cryst.* **39**, 391–400.
- Pallansch, M. & Roos, R. (2001). *Fields Virology*, 4th ed., edited by D. M. Knipe & P. M. Howley, ch. 24. Philadelphia: Lippincott Williams & Wilkins.
- Papageorgiou, N., Watier, Y., Saunders, L., Coutard, B., Lantiez, V., Gould, E. A., Fitch, A. N., Wright, J. P., Canard, B. & Margiolaki, I. (2010). *Z. Kristallogr.* **225**, 576–580.
- Pawley, G. S. (1981). *J. Appl. Cryst.* **14**, 357–361.
- Porter, A. G. (1993). *J. Virol.* **67**, 6917–6921.
- Probst, C., Jecht, M. & Gauss-Müller, V. (1998). *J. Virol.* **72**, 8013–8020.
- Racaniello, V. (2007). *Fields Virology*, 6th ed., edited by D. M. Knipe & P. M. Howley, pp. 795–838. Philadelphia: Lippincott Williams & Wilkins.
- Rhoades, R. E., Tabor-Godwin, J. M., Tsueng, G. & Feuer, R. (2011). *Virology*, **411**, 288–305.
- Rietveld, H. M. (1969). *J. Appl. Cryst.* **2**, 65–71.
- Romero, J. R. & Modlin, J. F. (2015). *Mandell, Douglas, and Bennett's Principles and Practice of Infection Diseases*, 8th ed., edited by J. E. Bennett, R. Dolin & M. J. Blaser, pp. 2066–2072. Philadelphia: Elsevier Saunders.
- Rossmann, M. G., Bella, J., Kolatkar, P. R., He, Y., Wimmer, E., Kuhn, R. J. & Baker, T. S. (2000). *Virology*, **269**, 239–247.
- Santti, J., Harvala, H., Kinnunen, L. & Hyypiä, T. (2000). *J. Gen. Virol.* **81**, 1361–1372.
- Seipelt, J., Guarné, A., Bergmann, E., James, M., Sommergruber, W., Fita, I. & Skern, T. (1999). *Virus Res.* **62**, 159–168.
- Shi, D., Nannenga, B. L., Iadanza, M. G. & Gonen, T. (2013). *Elife*, **2**, e01345.
- Tan, J., George, S., Kusov, Y., Perbandt, M., Anemüller, S., Mesters, J. R., Nordner, H., Coutard, B., Lacroix, C., Leyssen, P., Neyts, J. & Hilgenfeld, R. (2013). *J. Virol.* **87**, 4339–4351.
- Valmas, A., Magioudis, K., Fili, S., Norrmann, M., Schluckebier, G., Beckers, D., Degen, T., Wright, J., Fitch, A., Gozzo, F., Giannopoulou, A. E., Karavassili, F. & Margiolaki, I. (2015). *Acta Cryst.* **D71**, 819–828.
- Von Dreele, R. B. (2001). *Acta Cryst.* **D57**, 1836–1842.
- Von Dreele, R. B. (2003). *Methods Enzymol.* **368**, 254–267.
- Von Dreele, R. B. (2005). *Acta Cryst.* **D61**, 22–32.
- Von Dreele, R. B. (2007). *J. Appl. Cryst.* **40**, 133–143.
- Von Dreele, R. B., Stephens, P. W., Smith, G. D. & Blessing, R. H. (2000). *Acta Cryst.* **D56**, 1549–1553.
- Wang, H.-M. & Liang, P.-H. (2010). *Expert Opin. Ther. Pat.* **20**, 59–71.
- Weidman, M. K., Sharma, R., Raychaudhuri, S., Kundu, P., Tsai, W. & Dasgupta, A. (2003). *Virus Res.* **95**, 75–85.
- Weidman, M. K., Yalamanchili, P., Ng, B., Tsai, W. & Dasgupta, A. (2001). *Virology*, **291**, 260–271.
- Wlodawer, A. & Vondrasek, J. (1998). *Annu. Rev. Biophys. Biomol. Struct.* **27**, 249–284.
- Wright, J. P. (2004). *Z. Kristallogr.* **219**, 791–802.
- Yalamanchili, P., Datta, U. & Dasgupta, A. (1997). *J. Virol.* **71**, 1220–1226.
- Yalamanchili, P., Weidman, K. & Dasgupta, A. (1997). *Virology*, **239**, 176–185.
- Yin-Murphy, M. & Almond, J. W. (1996). *Medical Microbiology*, 4th ed., edited by S. Baron, ch. 53. Galveston: University of Texas Medical Branch.