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

Purification, crystallization and preliminary X-ray diffraction studies of the archaeal virus resolvase SIRV2

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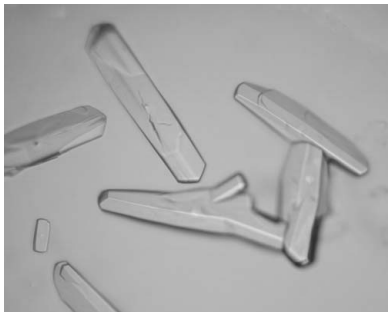
Received 17 March 2005
Accepted 13 April 2005
Online 22 April 2005



The Holliday junction (or four-way junction) is the universal DNA intermediate whose interaction with resolving proteins is one of the major events in the recombinational process. These proteins, called DNA junction-resolving enzymes or resolvases, bind to the junction and catalyse DNA cleavage, promoting the release of two DNA duplexes. SIRV2 Hjc, a viral resolvase infecting a thermophylic archaeon, has been cloned, expressed and purified. Crystals have been obtained in space group *C*2, with unit-cell parameters $a = 147.8$, $b = 99.9$, $c = 87.6$, $\beta = 109.46^\circ$, and a full data set has been collected at 3.4 Å resolution. The self-rotation function indicates the presence of two dimers in the asymmetric unit and a high solvent content (77%). Molecular-replacement trials using known similar resolvase structures have so far been unsuccessful, indicating possible significant structural rearrangements.

1. Introduction

Holliday junctions (four-way junctions) are universal intermediates in the repair and reorganization of DNA by homologous recombination. They are mobile links between two homologous DNA duplexes and generate new segments of heteroduplex DNA by branch migration. The crucial step is the final resolution of the junction without loss of nucleotides. Holliday junction-resolving enzymes mediate the termination of the process by recognizing DNA four-way junctions and introducing symmetrical nicks. The members of this ubiquitous family of structure-specific endonucleases function as dimers and require divalent cations for cleavage (reviewed in Lilley & White, 2001).

All of the archaeal genomes sequenced to date contain a gene encoding a Holliday junction-cleaving enzyme (Hjc) sequence. This small resolvase specifically recognizes and cleaves four-way DNA junctions through recognition of the particular three-dimensional structure. Two crystal structures of Hjc dimers have been reported so far (Bond *et al.*, 2001; Nishino *et al.*, 2001), both from archaea, revealing that this protein belongs to the nuclease family, with a folding motif clearly different from any other resolvase thus far known but similar to that of the type II restriction endonucleases. Recently, a Holliday junction resolvase was found in SIRV1 and SIRV2 (Birkenbihl *et al.*, 2001), two archaeal viruses infecting the extremely thermophilic archaeon *Sulfolobus islandicus* and both members of the *Rudiviridae* virus family. As a first step towards a detailed structural investigation of the first viral resolvase, we report in this paper the crystallization and preliminary X-ray analysis of the *S. islandicus* SIRV2 virus resolvase.

2. Expression, purification and DNA binding of SIRV2

The SIRV2 Hjc open reading frames were obtained by PCR reaction with the oligonucleotides SIRVC5 (GGGCCATGGAAATTAGACAATCCGGAAAAT) and SIRVB3 (GGGGGGATCCTTAGCTGTTAATTCCGTATT), digested with *Nco*I and *Hin*dIII and cloned into a modified pET24d expression vector with an upstream sequence coding for a His_6 tag followed by a TEV protease site (pETM11). The protein was expressed in *Escherichia coli* strain BL21 (DE3) Codon

Plus RIL (Stratagene, La Jolla, CA, USA), which contains a plasmid carrying extra genes for tRNAs, which are rare in *E. coli*. Cells were grown at 310 K in 6 l LB medium with 100 µg ml^{-1} kanamycin and 87 µg ml^{-1} chloramphenicol to an optical density at 600 nm of 0.6. After induction with 1 m*M* isopropyl thiogalactoside (IPTG; final concentration) cells were grown for a further 3 h. Cells were then harvested by centrifugation and resuspended in of 50 ml lysis buffer (50 m*M* Tris–HCl pH 8.0, 0.1 m*M* EDTA, 10% glycerol, 0.4 *M* ammonium sulfate). They were then disrupted using a French press and, after another 10 ml of lysis buffer had been added, lysates were cleared by centrifugation at 30 000*g* for 30 min. The resulting supernatant was diluted tenfold with buffer *A* (50 m*M* Tris–HCl pH 8, 50 m*M* NaCl, 10% glycerol). The supernatant was loaded onto a nickel–nitriloacetic acid bead column (Qiagen, Hilden, Germany) and elution was carried out as recommended by the manufacturer. The samples were further purified on a Superdex 75 (Amersham Biosciences Europe GmbH, Freiburg, Germany) column in gel-filtration buffer (50 m*M* Tris–HCl pH 7.0, 0.4 *M* ammonium sulfate, 15% glycerol) and concentrated by ultrafiltration to 10 mg ml^{-1} and kept at 277 K. This protocol led to greater than 98% pure samples as judged from Coomassie blue-stained gel.

Table 1
Diffraction data statistics.

Values in parentheses refer to the highest resolution shell.

Space group	*C*2
Unit-cell parameters (Å, °)	a = 147.8, b = 99.9, c = 87.6, β = 109.4
Resolution range (Å)	40–3.4
R_{sym} (%)	7.6 (32.8)
Completeness (%)	98.2 (98.9)
Average $I/\sigma(I)$	13.3 (2.3)
Redundancy	3.0

The SIRV2 resolvase activity was assessed by incubating 3 µl of protein at a concentration of 6 µ*M* for 15 min at 323 K in the presence of 3 µl of a synthetic short DNA four-way junction at 20 µ*M* and of 3 µl of a buffer consisting of 200 m*M* NaCl, 20 m*M* Tris–HCl pH 7.0, 15% glycerol and 150 m*M* ammonium sulfate (not shown). The DNA binding was analyzed by mixing SIRV2 and a DNA four-way junction in a buffer consisting of 120 m*M* NaCl, Tris pH 7.0, 10% glycerol, 0.2 m*M* DTT and 1 m*M* EDTA at room temperature. The result was visualized by gel-electrophoresis mobility assays on native gels: a 12% polyacrylamide gel (35:1 acrylamide:bisacrylamide) was run at room temperature in 45 m*M* Tris borate pH 8.3, 2 m*M* EDTA and revealed by ethidium bromide staining. Whereas no binding was observed in presence of magnesium, DNA junctions are shifted upon addition of SIRV2 resolvase without divalent cations (Fig. 1). However, we do not observe the formation of a homogenous SIRV2 dimer–DNA junction complex as required for optimal crystallization experiments. First, an excess of SIRV2 dimer is required to shift the entire DNA on the gel. In addition, we observed several high-molecular-weight species, indicating the probable formation of heterogenous multimers upon addition of resolvase.

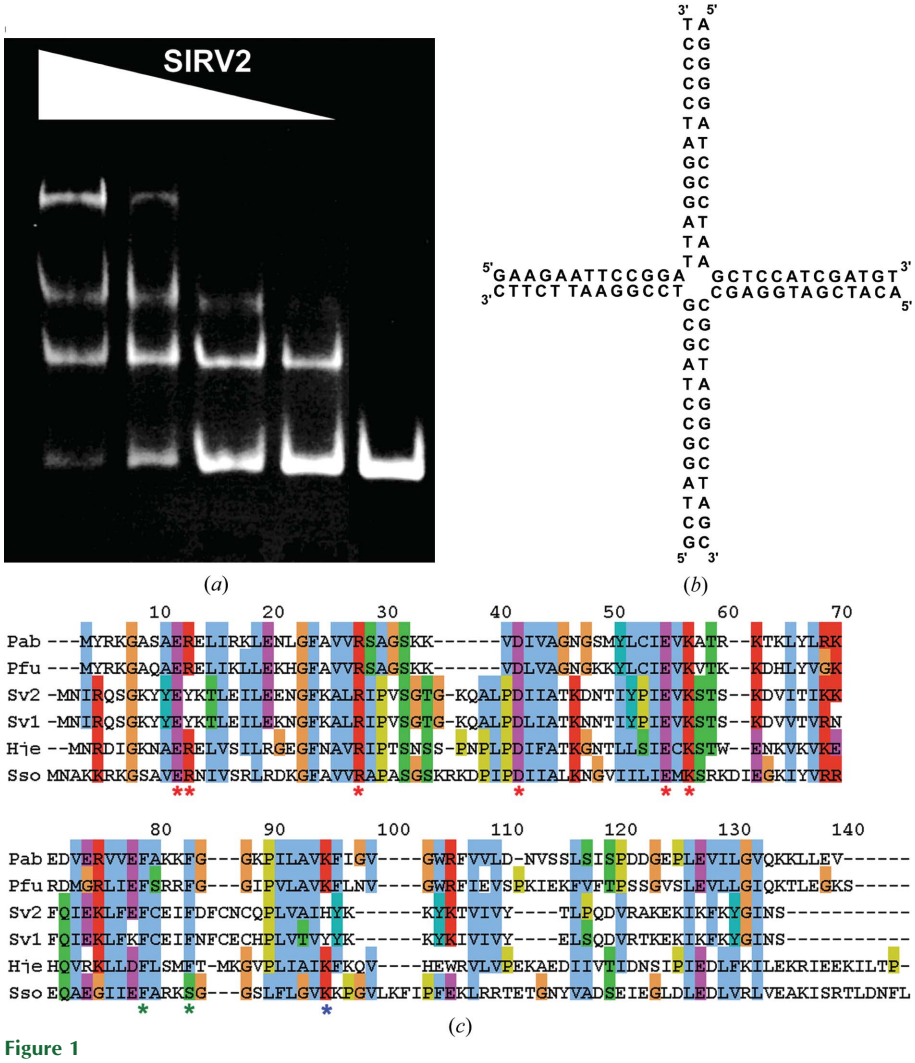


Figure 1
(*a*) Native polyacrylamide (12%) gel-shift experiment of a synthetic DNA four-way junction using increasing amounts of SIRV2 resolvases in the absence of divalent cations. The DNA concentration was 5 µ*M*. SIRV2 concentrations were (from right to left) 0, 2.5, 5, 7.5 and 15 µ*M*, respectively. (*b*) Synthetic DNA four-way junction used for gel-shift experiments. (*c*) Sequence alignment obtained using *ClustalW* (EMBL-EBI, Hinxton, UK). Essential residues for endonuclease activity, dimerization and DNA binding of Pfu-Hjc are indicated with red, green and blue stars, respectively (according to Komori *et al.*, 2000). Abbreviations used: Pab, *Pyrococcus abyssi* Hjc; Pfu, *P. furiosus* Hjc; Sv2, SIRV2 Hjc; Sv1, SIRV1 Hjc; Hje, *Sulfolobus solfataricus* Hje; SSo, *S. solfataricus* Hjc.

3. Crystallization

Crystallization trials were performed at 293 K using the sitting-drop method and a Mosquito nanolitre-drop crystallization robot (TTP Labtech, Melbourn, UK). Several commercial screens were used, mixing one volume of protein at 5 mg ml^{-1} in a buffer consisting of 50 m*M* Tris–HCl pH 7.0, 0.4 *M* ammonium sulfate, 15% glycerol with one volume of reservoir solution. Out of about 800 tested crystallization conditions, only one (30% PEG 550 MME, 0.1 *M* HEPES pH 8.2) led to the growth of small platelet crystals. Efforts to optimize this crystallization condition remained unsuccessful. Crystals with a suitable size for

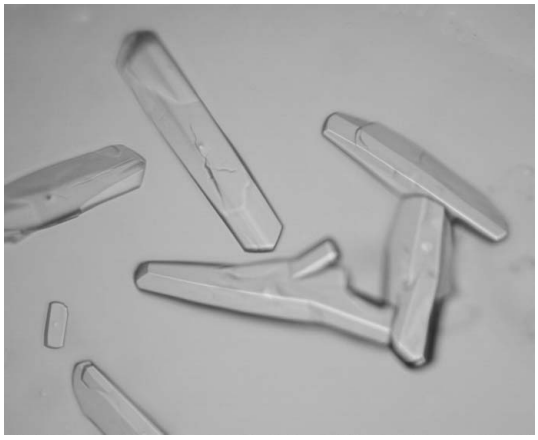


Figure 2
Picture of typical SIRV2 crystal platelets (maximum 400 µm in length).

data collection were obtained from 2 µl drops reproduced in Linbro plates (Fig. 2).

Handling of crystals was extremely difficult owing to their excessive fragility. Therefore, crystals were preferably reproduced in hanging drops to ease manual handling and to avoid their attachment to the bottom of the microbridge. Prior to data collection, crystals were cryoprotected for ∼30 min in a reservoir solution supplemented with 15% glycerol and flash-frozen in liquid ethane.

4. X-ray data collection and analysis

A full data set was collected at 90 K from one crystal on beamline ID29 at the ESRF (Grenoble, France). Data were processed using the *HKL* package (Otwinowski & Minor, 1996; see Table 1). The space group is *C*2, with unit-cell parameters a = 147.8, b = 99.9, c = 87.6 Å, β = 109.4°, and the crystal diffracted anisotropically to 3.0 Å. The calculated Matthews coefficients are compatible with the presence of two (V_M = 5.4 Å^3 Da^{-1}) dimers in the asymmetric unit with a solvent content of 77%, or four dimers (V_M = 2.7 Å^3 Da^{-1}) per asymmetric unit with a solvent content of 55%. The self-rotation function calculated using the 30–4.0 Å resolution range and a radius of integration of 35 Å (estimated radius for a dimer) shows the presence of two strong twofold non-crystallographic axes in the κ = 180° section (Fig. 3), revealing a pseudo-orthorhombic arrangement of molecules. As no significant peak corresponding to a possible translation was observed in a native Patterson, the presence of two dimers in the asymmetric unit is more likely and would explain the extreme fragility of crystals given the high solvent content.

Extensive attempts at solving the structure by molecular-replacement performed using available Hjc models (PDB codes 1gef and 1ipi) and various software packages (*AMoRe*, *MOLREP*, *CNS*, *CaspR*) remained unsuccessful. This was quite unexpected given the rather high sequence similarity of SIRV2 to already solved Hjc structures and may therefore be an indication that some significant changes in the structure occur compared with Hjc. Interestingly, Hje, another small archaeal resolvase of the same Hjc family, was recently crystallized and led to similar molecular-replacement difficulties (Middleton *et al.*, 2003). The Hje structure was eventually solved by MAD, revealing significant structural rearrangements compared with Hjc (Middleton *et al.*, 2004). Further molecular-replacement attempts

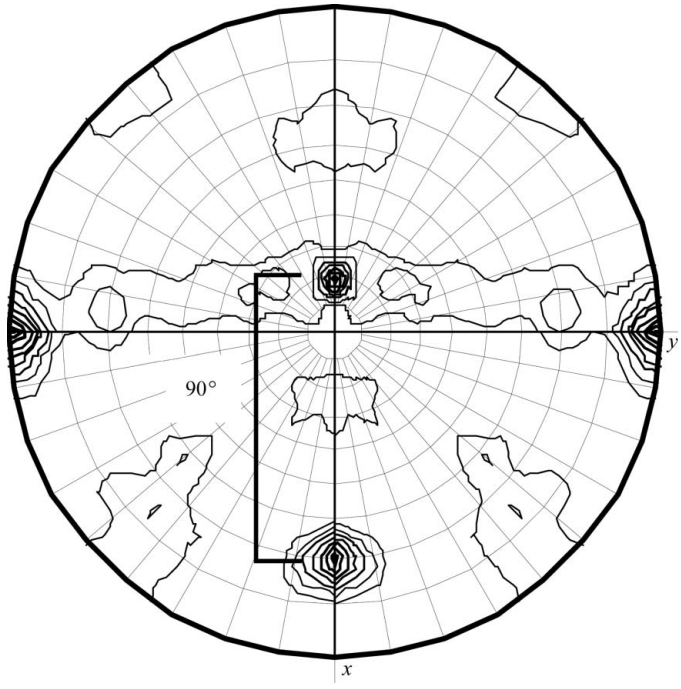


Figure 3
Self-rotation function for SIRV2 using *MOLREP* in the 30–4.0 Å resolution range and using a 35 Å integration radius. In addition to the crystallographic axis, two strong peaks are observed at 90° from each other for κ = 180°.

to solve the SIRV2 structure were performed using Hje as a model (PDB codes 1ob8 and 1ob9), but without success. These two cases of molecular-replacement difficulties with highly similar proteins emphasize the well known flexibility of resolvases, as previously observed for the phage T4 endo VII resolvase (Raaijmakers *et al.*, 2001). Improvements of the diffraction limit using gel techniques, as well as site-directed mutagenesis and introduction of several methionines for experimental phasing by MAD with selenomethionines, are currently under way to determine the structure.

This work was supported by an EMBO long-term fellowship attributed to EE. The authors would like to thank the ID-29 beamline staff at the ESRF (Grenoble, France) for their support.

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