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Functional expression, monodispersity and conformational changes in the SBMV virus viral VPg on binding TFE

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ABSTRACT

Southern bean mosaic virus (SBMV) RNA purified from infected plants was used for cloning the viral genome-linked protein (VPg) and was subsequently expressed in *Escherichia coli*. Circular dichroism (CD), dynamic light scattering (DLS) and saturation transfer difference (STD) by nuclear magnetic resonance (NMR) measurements were employed to determine the degree of monodispersity and to investigate the conformational changes in the absence and presence of trifluoroethanol (TFE) which indicated increased helical content with increasing concentration of TFE. 8-Anilino-1-naphthalenesulfonic acid (ANS) was used as a probe to compare the unfolding regions of the protein before and after addition of TFE. The results indicated that although the TFE concentration influences VPg folding, it does not play a role in nucleotide binding and that the local solvent hydrophobicity causes significant conformational changes.

1. Introduction

Genome-linked viral proteins (VPgs) are involved in a number of processes ranging from replication to viral protein synthesis [1,2]. VPgs are small proteins that are covalently linked to the 5′ end of viral RNA via a phosphodiester bond formed between the hydroxyl groups of amino acid residues and the 5′ phosphate groups of RNA [3,4]. Often encountered in viruses with single-stranded positive-sense RNA (ssRNA) genomes, VPgs from fungal viruses, plant viruses and animal viruses with double or positive single strand (ssRNA) have been characterized [5,6]. VPgs from plant and animal viruses share many features, for example, they are products of polyprotein processing and are uridylated by their cognate RNA-dependent RNA polymerase (RdRP) [7–9] enabling VPgs to operate as primers during viral RNA synthesis. VPgs also share the presence of high percentages of basic amino acids (mostly lysine, glycine, threonine and arginine) contributing to the interaction with the negatively charged RNA [10]. The covalent binding of VPgs to RNAs exhibits some differences; picornaviruses, potyviruses and caliciviruses use the hydroxyl group of a tyrosine residue whereas comoviruses and nepovirus are reported to use a serine residue [6]. Threonine also contains a hydroxyl group, but the only evidence that it is used for RNA binding was reported by [11] when investigating VPg from SBMV covalently attached to genomic RNAs. VPg from sobemovirus is a cleavage product of its precursor polyprotein (VPg-proteinase-polymerase) [12] and the residue for RNA binding is not conserved within its genre [13]. All positive-sense ssRNA viruses that infect mammalian, insect or plant cells replicate in association with host endomembrane [14] and different host factors may influence the process [15,16]. Some phytoviral (*Sesbania mosaic virus*; *Potato virus A*, *Potato virus Y*, *Lettuce mosaic virus*, and *Rice yellow mottle virus*) VPgs were shown to be natively unfolded proteins [17–20] and hence recalcitrant for crystallization. Therefore, to date, not a single crystal structure of these proteins has been determined. Nevertheless, the crystal structures of synthetic peptides corresponding to *Picornaviridae* VPgs (<3 kDa) (PDB: 2D7S, 4IKA, 3CDW) complexed with their cognate RdRP indicate that the peptides are almost completely random coiled without any alpha helix or beta strand. More recently, the solution structure of recombinant VPg from *Caliciviridae* (PDB: 2MXD) has been elucidated [21].

In the present study, VPg from SBMV was expressed in *Escherichia coli* and purified. Circular dichroism (CD) was employed to assess the VPg secondary structure conformational content and its variation in the presence of increasing concentrations of reagents that mimic membrane environments

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(2,2,2-trifluoroetahnol, TFE). Far UV-CD spectra and 8-anilino-1-naphthalenesulfonic acid (ANS) were used to compare the unfolded regions of the protein before and after the addition of TFE and saturation transfer difference by nuclear magnetic resonance (STD-NMR) spectroscopy was employed to compare the binding pattern between the VPg from SBMV and dNTPs, to gain insights into molecular recognition of different nucleotides by VPg.

2. Methods

2.1. Virus purification and RNA extraction

The virus was purified from infected leaves of *Phaseolus vulgaris* following the method of [22]. Viral RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions.

2.2. Primer design, cDNA syntheses and PCR amplification of the ORF1

Primers were designed as shown below to amplify the sequence that encodes VPg from genomic RNA of an isolate of SBMV. *Bam*HI and *Xho*I restriction sites were incorporated in the forward and reverse primers, respectively, to facilitate cloning in pET28a expression vector. A TEV protease cleavage site was also incorporated in the forward primer to permit cleavage of the hexahistidine tag of the recombinant protein after purification.

Forward primer: 5′-GGATCC**GGTGAAAATTTATATTTTCAA**GGTACTCTACCT CCTGATCTGTCCG-3′ (*Bam*HI restriction site underlined and TEV cleavage site in bold).

Reverse primer: 5′-CTCGAGTCATTCCTGAGCTGAAGTCCA-3′ (*Xho*I restriction site underlined).

First strand cDNA was synthesized using Superscript II Reverse Transcriptase (Invitrogen). Polymerase chain reaction (PCR) was performed for the amplification of the sequence that encodes VPg in 100 μL mixture containing 50 ng of genomic RNA, 1 μL (10 μM) of each primer, 2 μL (10 mM) dNTPs, 10 μL of PCR buffer (100 mM Tris–HCl, pH 8.8, 500 mM KCl, 0.8% Nonidet P40, and 25 mM $MgCl_2$), and 2.5 U of native *Taq* DNA polymerase enzyme (MBI Fermentas). The reaction was carried out using the following reaction cycles in a programmable thermocycler (Eppendorf): initial denaturation at 95 °C for 10 min followed by 30 consecutive cycles of denaturation at 95 °C for 30 s, annealing for 1 min at 55 °C, extension at 72 °C for 1 min 30 s, followed by a final extension at 72 °C for 10 min. The amplification product was analyzed by electrophoresis on a 1% agarose gel stained with ethidium bromide. The specific PCR product obtained was purified using PCR gel purification kit (Qiagen) and the product was used for ligation in pGEM-T Easy vector.

2.3. Cloning in pGEM-T easy vector

In order to clone the sequence that encodes VPg, pGEM-T Easy vector (Promega) was used. The resulting PCR product and pGEM-T Easy vector were ligated overnight at 4 °C using T4 DNA ligase. This construct was transformed into *E. coli* DH5α cells and the resulting colonies were screened by blue white colony selection and PCR. One clone was used to plasmid extraction and it was sequenced by automatic sequencer ABI 377 DNA Sequencer.

2.4. Cloning in pET28a

The sequence cloned in pGEM-T Easy vector was digested with *Bam*HI and *Xho*I restriction enzymes and analyzed on a 1% agarose gel. A 300 bp insert (ORF) was purified from the agarose gel by using a gel extraction and purification kit (Qiagen) and cloned in pET28a vector (Novagen). Positive clones were first selected by PCR and reconfirmed by restriction digestion.

2.5. Expression of recombinant VPg in E. coli using pET28a vector

For expression, VPg protein was tagged with 6xHis; *E. coli* cells BL21-CodonPlus®-RIL were transformed with the pET28a construct and incubated in LB broth (with 0.2% glucose and 34 μg/ml kanamycin for selection). The medium was inoculated with an overnight culture (1:100 dilution) and the culture was incubated under agitation at 30 °C until an OD_{600} of ~0.5 was attained. Subsequently, 0.2 mM IPTG was added and the culture was further incubated at 18 °C for 16 h.

2.6. Protein purification

2.6.1. Purification of the VPg 6xHis recombinant protein

Cells were harvested by centrifugation at 6000 × *g*, at 4 °C for 20 min and lysed in buffer 20 mM sodium phosphate pH 7.4, 200 mM NaCl (buffer A) by sonication on ice. The clear supernatant obtained by centrifugation at 15,000 × *g* for 45 min (4 °C) was subsequently applied onto a nickel resin column pre-equilibrated with buffer A. The column was washed with buffer A which additionally contained 70 mM imidazole and the recombinant protein was eluted with buffer A which contained 400 mM imidazole, concentrated and subjected to a final step of molecular exclusion chromatography by utilizing a Superdex G75/300 GE column. The purity of VPg throughout *E. coli* expression and purification steps was determined by SDS-PAGE.

2.7. Dynamic light scattering

DLS measurements were carried out using freshly prepared samples in buffer 20 mM sodium phosphate pH 7.5, 100 mM NaCl. Each experiment was carried out in a quartz cuvette with an optical path length of 3 mm at 25 °C and the results presented are the average values obtained from 20 scans. The experiments were repeated at different pHs in the presence of salts, amino acids, sugars, detergents, reducing agents and also in the presence of possible ligands, such as, dATP, dUTP, dGTP and dCTP (Table 1).

2.8. Circular dichroism spectroscopy

Far UV-CD spectra were recorded at room temperature (25 °C) on a Jasco J-710 Spectropolarimeter (Jasco, Tokyo, Japan) and quartz cells with a path length of 0.5 mm. CD spectra were recorded in the 190–260 nm range at a scan rate 50 nm/min, response time of 1.0 s, spectral bandwidth of 1.0 nm and spectral resolution of 0.1 nm. For each spectrum, 7 accumulations were performed. The VPg concentration was maintained constant at 30 μM during all experiments. The addition of 2,2,2-trifluoroethanol (TFE, Sigma) was performed in the 0–30% range with an increment of 10% (v/v). All spectra were corrected by subtraction of the respective buffer spectra. Secondary structure percentages for each tested condition were calculated with CONTINLL software of CDPro package, using the reference set of proteins SMP56 [23].

2.9. Binding of probe 8-aniline-1-naphthalene sulfonate (ANS)

The fluorescence measurements were performed at room temperature (25 °C) by using an ISS PC1 steady-state Spectrofluorimeter (Champaign, IL, USA) equipped with quartz cells of 10 mm path lengths. Both excitation and emission bandwidths were set at 8 nm. The excitation wavelength at 370 nm was chosen since it only causes excitation of ANS. The emission spectrum was collected in the range of 390–700 nm with an increment of 1 nm and each point