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Characterization of the single-stranded DNA binding protein pV$^{VGJ\Phi}$ of VGJΦ phage from *Vibrio cholerae*

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ABSTRACT

pV$^{VGJ\Phi}$, a single-stranded DNA binding protein of the vibriophage VGJΦ was subject to biochemical analysis. Here, we show that this protein has a general affinity for single-stranded DNA (ssDNA) as documented by Electrophoretic Mobility Shift Assay (EMSA). The apparent molecular weight of the monomer is about 12.7 kDa as measured by HPLC–SEC. Moreover, isoelectrofocusing showed an isoelectric point for pV$^{VGJ\Phi}$ of 6.82 pH units. Size exclusion chromatography in 150 mM NaCl, 50 mM sodium phosphate buffer, pH 7.0 revealed a major protein species of 27.0 kDa, suggesting homodimeric protein architecture. Furthermore, pV$^{VGJ\Phi}$ binds ssDNA at extreme temperatures and the complex was stable after extended incubation times. Upon frozen storage at −20 °C for a year the protein retained its integrity, biological activity and oligomericity. On the other hand, bioinformatics analysis predicted that pV$^{VGJ\Phi}$ protein has a disordered C-terminal, which might be involved in its functional activity. All the aforementioned features make pV$^{VGJ\Phi}$ interesting for biotechnological applications.

1. Introduction

The single-stranded DNA binding protein (SSB) of phage VGJΦ was previously identified to be encoded by ORF112 [1] and named pV$^{VGJ\Phi}$ [2]. This protein, isolated to homogeneity, binds ssDNA but not dsDNA [2]. However, a deeper biochemical characterization of this protein is necessary to rule out its biological function.

A previous mass spectrometry analysis showed a peptide sequence of a predicted molecular weight of 12.72 kDa, which corresponds to the predicted amino acid sequence of the open reading frame ORF112 VGJΦ [1], without the N-terminal methionine. Accordingly, pV$^{VGJ\Phi}$ was prominently expressed in *V. cholerae* infected with VGJΦ [1].

SSB proteins are characterized by the presence of a central conserved oligosaccharide/oligonucleotide binding (OB) fold comprising 5 antiparallel β-sheets that selectively bind ssDNA in a cooperative and non-sequence specific manner [3]. OB domains bind ssDNA in a cleft formed primarily by β-strands, by using aromatic residues that stack against nucleotide bases, and positively charged residues that form ionic interactions with DNA backbone [4]. The N- and C-terminal regions of the protein support effective interactions among functional entities of the SSB and other cell proteins involved in DNA metabolism, just to guarantee their important role in DNA replication, recombination and repair [5]. SSB proteins protect DNA from nucleases, stabilize ssDNA intermediates that are generated during DNA processing and prevent formation of DNA secondary structures [6]. Due to this array of essential functions SSB proteins are found in all living organisms and in viruses.

SSB proteins have found numerous applications in analytical molecular biology methods. They have been used to increase amplification efficiency with a number of diverse templates, prevent and reduce primer dimer formation, one of the problems known to cause inhibition of primer hybridization to the template and reduction of the number of primer molecules available for annealing [7]. They also interact efficiently with RNA, allowing a dramatic increase in the size of the cDNA synthesised by the reverse transcriptase activity of T. Termophilus DNA polymerase [8]. Provided the relevant biological role of SSBs and their broad spectrum of applications, a biochemical characterization of pV$^{VGJ\Phi}$ is a novel and valuable result. This paper shows the oligomeric state, the ssDNA-protein reaction kinetics and the effect of temperature on the DNA–protein interaction as well as stability of purified pV$^{VGJ\Phi}$ upon storage at −20 C for a year.

2. Materials and methods

2.1. Isolation and purification of pV$^{VGJ\Phi}$

pV$^{VGJ\Phi}$ was obtained from *Vibrio cholera* and purified under denaturing conditions as previously described [2]. SDS-PAGE (15% polyacrylamide) was used to monitor the process and the protein was quantified by Lowry. Native PAGE was run in a separating gel having a

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discontinuous polyacrylamide concentration gradient cast of 6 and 10%. The molecular size and degree of protein purity were estimated from densitometric scans of Coomassie brilliant blue stained gels, using a GENE GENIUS Gel Documentation System (Syngene Synoptics Ltd, Cambridge, UK).

2.2. Kinetics of $pV^{VGJ\Phi}$-ssDNA complex formation

DNA binding activity of the protein was assayed by EMSA as described before [2]. The time course of $pV^{VGJ\Phi}$ ssDNA complex formation was carried out by setting up five independent reactions with 23 μg of $pV^{VGJ\Phi}$ and 0.5 μg of VGJΦ ssDNA at room temperature, 5 min from one each other, and analysed by agarose gel electrophoresis (0.5%) at 0, 5, 10, 15 and 20 min. Ethidium bromide stained gels were documented in a GENE GENIUS gel system (Syngene Synoptics Ltd, Cambridge, UK).

2.3. DNA binding activity of $pV^{VGJ\Phi}$ at extreme temperatures

Binding reaction mixtures containing 23 μg of $pV^{VGJ\Phi}$ and 0.5 μg of VGJΦ ssDNA were incubated at 80 °C and 94 °C for different intervals as follows**:** 20, 40, 60, 80, 100, 110 and 120 min for 80 °C and 5, 10, 15, 20, 25, 30, 35 and 40 min for 94 °C. Samples were analysed by agarose gel electrophoresis (0.5%) against a control reaction having only ssDNA without $pV^{VGJ\Phi}$, kept for 120 and 40 min at 80 and 94 °C, respectively. Electrophoresis gels were documented as before**.**

2.4. High performance liquid chromatography–size exclusion chromatography (HPLC–SEC)

Approximately 100 μg of the purified $pV^{VGJ\Phi}$ was injected via a V-7 valve, using a 100 μl loop at 25 °C on a Superdex 200 HR 10/30 column (former Amershan Pharmacia Biotech, code 17-1088-01) with a size exclusion limit of 1300 kDa and an effective separation range between 10 and 600 kDa. The column was attached to a LKB liquid chromatograph (Pharmacia Biotech Sweden) coupled to a Shimadzu data processor (C-R6A CHROMATOPAC). 150 mM NaCl, 50 mM phosphate buffer, pH 7.0 were used as mobile phase and delivered at a constant flow rate of 0.4 ml min^{-1}. The column effluent was monitored at A_{280} nm. Protein molecular weight determination was performed by using migration distances of the Conalbumin (75 kDa), ovalbumin (43 kDa), carbonic anhidrase (29 kDa), ribonuclease A (13.7 kDa) and aprotinin (6.5 kDa) used as molecular mass standards (GE Healthcare, UK); and as a high molecular mass standard, aldolase (158 kDa) was used.

2.5. Isoelectric focusing

Isoelectric focusing was carried out on non-denaturing slab gels of 12.5% polyacryalmide containing ampholines (pH 3.0 to 9; Pharmacia). A total of 100 μg of proteins was run at 600 V/h for 45 min. The pH gradient was calibrated by using an Isoelectric Focusing Calibration Kit Broad pI (pH 3–10) (GE Healthcare, UK Limited). Gels were fixed in 20% trichloroacetic acid–30% methanol–3.5% sulfosalicylic acid and silver stained, according to the manufacturer's recommendations, using the GE Health Care standard focusing equipment. Six replicas of the experiment were run and the gel was analyzed in a GENE GENIUS gel system (Syngene Synoptics Ltd, Cambridge, UK).

2.6. Protein stability assay

Purified $pV^{VGJ\Phi}$ protein was concentrated up to 2.3 mg ml^{-1} and filter-sterilized through a 0.22 μm pore-size membrane. The sample in 20 mM Tris–HCl pH 8.0 was then divided into aliquots with different additives: 0.2 M NaCl; 0.2 M NaCl plus 50% glycerol; 0.2 M NaCl plus 0.2% triton X-100 and 0.2 M NaCl plus 50% glycerol and 2 mM EDTA. An aliquot without additive was used as control. The final protein concentration was 1.5 mg ml^{-1}. Finally, samples were stored at −20 °C and 20 μg of $pV^{VGJ\Phi}$ was assayed at 0, 4 and 12 months by EMSA, SDS-PAGE and Native-PAGE as described earlier for ssDNA binding activity and integrity.

2.7. Bioinformatics analysis

The aminoacidic sequence of $pV^{VGJ\Phi}$ protein was submitted via web to PredictProtein server, an Internet service that searches up-to-date public sequence databases, creates alignment and predicts aspects of protein structure and function [9]. This server uses softwares to predict residue solvent accessibility (PROFacc) and secondary structure (PROFsec) for reliably scored residues only [10]. Moreover, the program ISIS (Interaction Sites Identified from Sequence) predicts specifically residues involved in external protein–protein interactions [11]. Programs like PROFbval, Ucon and MD predict different aspects of disorder: PROFbval is a neural-network-based method that predicts flexible and rigid residues in proteins [12]. Ucon combines predictions for protein-specific contacts with a generic pair wise potential for unstructured regions [13] and MD is a novel META-Disorder prediction method that molds various sources of information predominantly obtained from orthogonal prediction methods, just to improve significantly performance over its constituents [14].

3. Results

3.1. Molecular weight and isoelectric point determination of $pV^{VGJ\Phi}$

The molecular weight of $pV^{VGJ\Phi}$ was determined by HPLC–SEC into a Superdex 200 HR 10/30 column. Only three peaks were observed. The major peak was eluted at 46.3 min (Fig. 1a) with a calculated molecular weight of 27 kDa. This value corresponds closely to the expected MW of the dimeric form of the predicted gene $V^{VGJ\Phi}$ product (25.44 kDa). Also, another minor peak was detected at 50.9 min with an estimated molecular weight of 6.66 kDa, which agrees with half of monomer molecular mass. The appearance of this peak could be explained by assuming a little misfolding of the protein in the refolding process, performed under the denaturing purification conditions [2].These data suggest that purified $pV^{VGJ\Phi}$ protein, like the majority of SSBs from bacteriophages occurs mostly as a homodimer [15,16], which is usually the functional structure in filamentous bacteriophages [17,18].

Isoelectric focusing of purified $pV^{VGJ\Phi}$ produced a main band with a pI estimated at 6.82 that matches well with the NTI vector suite 6 program (InforMax, Inc.) predicted value of 6.52.

3.2. Oligomeric state of $pV^{VGJ\Phi}$

Fig. 1 shows that purified $pV^{VGJ\Phi}$ protein migrated as a single band (Fig. 1b) in SDS-PAGE, while in the native-PAGE two bands were clearly identified (Fig. 1c, lane 1). The major species was localized in the 6% region, which represents $pV^{VGJ\Phi}$ dimmer. This result was verified after applying to PAGE, the pooled and concentrated samples of the major HPLC–SEC peak, which migrated as a single band in 6% region of the separating gel (Fig. 1c, lane 2). The minor peak of 6.66 kDa was localized in the 10% region of the polyacrylamide gradient gel. We think it could be due to the little fraction of misfolded protein obtained during refolding process, since it migrated more than the monomer into 10% region of gel, as evidenced by native-PAGE of heat denatured sample (Fig. 1c, lane 3).

3.3. $pV^{VGJ\Phi}$ activity under different conditions

Purified $pV^{VGJ\Phi}$ protein bound ssDNA of VGJΦ, from an effective concentration of 0.03 μg μl^{-1} up to an apparent saturation of 0.5 μg μl^{-1}.