BIOCHEMICAL CHARACTERIZATION OF THE DNA BINDING PROTEINS OF THE PHAGES VGJΦ AND CTXΦ OF VIBRIO CHOLERAE.

CARACTERIZACIÓN BIOQUÍMICA DE LAS PROTEÍNAS DE UNIÓN A ADN DE LOS FAGOS VGJΦ Y CTXΦ DE VIBRIO CHOLERAE.

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BIOCHEMICAL CHARACTERIZATION OF THE SINGLE-STRANDED DNA BINDING PROTEINS OF THE PHAGES VGJΦ AND CTXΦ OF VIBRIO CHOLERAE.

ABSTRACT. The DNA binding proteins pVVGJΦ and pVCTXΦ of the vibriophages VGJΦ and CTXΦ localize to the soluble fraction and insoluble, respectively, of the cell lysates of Vibrio cholerae infected with VGJΦ and recombinant E. coli lysates. Both proteins were purified to homogeneity and obtained biologically active to carry out their biochemical characterization. The apparent molecular weight of monomeric pVVGJΦ is about 12.7 KDa and 16.8 kDa for pVCTXΦ, as measured by SDS-PAGE. Isoelectrofocusing showed a pI for pVVGJΦ of 6.82 pH units while pVCTXΦ exhibited a value of 6.47 pH units. Size exclusion chromatography in NaCl, 150 mM; Sodium phosphate, 50 mM; pH 7.0 revealed a major protein species of 27.0 kDa, suggesting homodimeric protein architecture for pVVGJΦ protein and a major peak of 65 kDa for pVCTXΦ, showing a homotetrameric state for this protein. Biological activity was assayed by electrophoretic mobility shift assays (EMSA). The pVVGJΦ protein is a single stranded DNA binding protein, while the pVCTXΦ protein is an unspecific DNA binding, due to it was able to bind ssDNA as well as dsDNA substrates. The influence of binding reaction times from 0 to 20 min on protein-DNA complex formation was examined. Both proteins were found to almost instantly bind the VGJΦ ssDNA. Once the fully saturated complex was formed, it remained apparently unchanged upon further prolonged incubations of up to 20 min.

Key words: Vibrio cholerae, single stranded DNA, double stranded DNA, VGJΦ phage, pVVGJΦ protein, CTXΦ phage, pVCTXΦ protein.

RESUMEN. Las proteínas de unión a ADN pVVGJΦ y pVCTXΦ de los vibriofagos VGJΦ y CTXΦ se localizan en la fracción soluble e insoluble, respectivamente, de los lisados celulares de Vibrio cholerae infectados con VGJΦ y E. coli recombinante. Ambas proteínas fueron purificadas a homogeneidad y obtenidas biológicamente activas para realizar su caracterización bioquímica. El peso molecular aparente de los monómeros es alrededor de 12.7 KDa para pVVGJΦ y 16.8 kDa para pVCTXΦ según resultados de SDS-PAGE. El punto Isoeléctrico obtenido para pVVGJΦ fue 6.82 unidades de pH, mientras que pVCTXΦ exhibió un valor de 6.47 unidades de pH. La cromatografía de exclusión molecular en NaCl, 150 mM; fosfato de sodio, 50 mM; pH 7.0 reveló una proteína de 27.0 kDa, sugiriendo una arquitectura homodimérica para la proteína pVVGJΦ y un pico mayoritario de 65 kDa para la proteína pVCTXΦ, mostrando un estado homotetramérico para esta proteína. La actividad biológica fue realizada por ensayos de variación de la movilidad electroforética. La proteína pVVGJΦ exhibió una actividad de retardo específica para el ADN de simple cadena (ADNsc) de VGJΦ, mientras pVCTXΦ fue capaz de unirse a ambos sustratos (ADNsc y ADNdc). La cinética de reacción en la formación del complejo proteína-ADN fue estudiada de 0 a 20 min. Se encontró que ambas proteínas se unieron casi instantáneamente al ADNsc del fago VGJΦ. Después que el complejo completamente saturado fue formado, permaneció aparentemente incambiable, después de incubación prolongada hasta 20 min.

Palabras clave: Vibrio cholerae, ADN simple cadena, ADN doble cadena, fago VGJΦ, proteína pVVGJΦ, fago CTXΦ, proteína pVCTXΦ.
INTRODUCTION

Cholera is a diarrheal disease caused in humans by infection with the gram-negative bacterium *Vibrio cholerae* expressing the pathogenic factors cholera toxin and the toxin co regulated pilus (1). Several filamentous vibriophages mediate the horizontal transfer of these and other pathogenic genetic traits within this species (2-7). The most studied CTXΦ phage plays an important role for *V. cholerae* pathogenicity, since it encodes the cholera toxin genes (3). VGJΦ phage was more recently discovered and found to exert specialized transduction of the CTXΦ genome, having the potential for virulence conversion of non-pathogenic strains of *V. cholerae* (8;9). Therefore, the molecular biology of these phages is of much interest to the community of vibriologists.

The genomes of VGJΦ and CTXΦ carry the putative homologous open reading frame frame 112 (orf112) and rstB genes, respectively, which encoded DNA binding proteins (SSBs), named pVVGJΦ and pVCTXΦ (10). pVVGJΦ is a single stranded DNA binding protein, while the pVCTXΦ protein is a unspecific DNA binding due to it was able to bind ssDNA as well as dsDNA substrates Both proteins were purified at homogeneity to study their biological activity, which paved the way for their further biochemical characterization.

This work describes the biochemical characterization performed to the pVVGJΦ and pVCTXΦ proteins. Here we show different biochemical and physical parameters in terms of isoelectric pH, oligomeric state, and the DNA-protein reaction kinetics.

MATERIALS AND METHODS

PROTEIN METHODS

*Vibrio cholerae* strain 569B (O1 serogroup, Inaba serotype, Classical biotype) infected with the filamentous phage VGJΦ (9) was the source of the phage’s SSB, termed pVVGJΦ. Purification of the soluble pVVGJΦ from the cytoplasmic content of the bacteria was conducted as in our previous report (10) for the formerly known Orf112.

*Escherichia coli* Top 10 was used to obtain the recombinant pVCTXΦ as described previously (10). The pVCTXΦ was purified according to purification protocol described before (10).

Samples of the pVVGJΦ and pVCTXΦ proteins were concentrated when required by ultrafiltration to achieve the desired concentration. An Amicon stirred ultrafiltration cell model 8050 (Amicon, Inc. Beverly, M.A.), fitted with a 10 kDa molecular weight cut off (MWCO) membrane was used for sample volumes greater than 5.0 ml and Centrisart tubes (Sartorius GmbH, Germany) with 10 kDa MWCO membranes for smaller samples. Protein concentration was estimated by the method of Lowry with bovine serum albumin (Sigma, A7888) as the standard reagent.

Proteins were profiled by analytical SDS-PAGE in an EC120 mini vertical gel system (Fisher Scientific, No.: 1202ECA-115) according to the Laemmli’s procedure in a 15% polyacrylamide vertical gel (height × width × depth: 7 × 8 × 0.075 cm) at 25°C with a constant current intensity of 15 mA and free voltage until the bromophenol blue dye migrated off the gel.

The molecular size and the 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).

DNA BINDING ACTIVITY OF pVVGJΦ

DNA binding activity of the protein was assessed by EMSA as described before (10). Briefly, the binding reaction consisted of 0.5 μg of DNA mixed with pVVGJΦ or pVCTXΦ protein, in appropriate amounts in 20% glycerol, 0.25 mM EDTA, 0.3 μM bovine serum albumin and 20 mM Tris-HCl, pH 8.0. The mix was incubated for 20 minutes at room
temperature and the reaction products were analyzed by 0.5% agarose gel electrophoresis like in the previous report (10). The time course of protein-DNA complex formation was performed by setting up five independent binding reactions with about 23 μg of pVVGJΦ or pVCTXΦ and 0.5 μg of VGJΦ ssDNA at room temperature spaced 5 minutes each from the other so that they could be checked by 0.5% agarose gel electrophoresis immediately after set up and after 5, 10, 15 and 20 minutes. DNA-binding competition assays were performed with a constant amount of genomic ssDNA of VGJΦ (400 ng) and increasing amounts (500 and 1,000 ng) of a non related dsDNA (sheared calf thymus). The ethidium bromide stained gels were documented in a GENE GENIUS gel system (Syngene Synoptics Ltd, Cambridge, UK).

**HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-SIZE EXCLUSION CHROMATOGRAPHY (HPLC-SEC).**

One aliquot containing 100 μg of the purified protein was loaded 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). The mobile phase consisted of 150 mM NaCl, 50 mM phosphate buffer, pH 7.0 was delivered at a constant flow rate of 0.4 ml·min⁻¹ (back-pressure: 1 Mρ). The column effluent was monitored at 280 nm. The samples were injected via a V-7 valve, using a 100 μL loop. The molecular weight determination of the protein was made as recommended (GE Healthcare, UK), using the migration distances of native marker proteins (Low Molecular Weight Kit) and of Blue Dextran 2000 (void volume determination). Conalbumin (75 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa) and aprotinin (6.5 kDa) were used as the molecular mass standards (GE Healthcare, UK); aldolase (158 kDa) was also used as a high molecular mass standard. The Kav values for the Calibration Kit proteins were calculated using the equation: Kav = Ve-Vo/Vc-Vo; where Vo is the column void volume, Ve is the elution volume and Vc is the geometric column volume. Standard curve was generated by plotting Kav versus log Mr for known molecular weight standards.

**ISOELECTRIC FOCUSING.**

Isoelectric focusing was carried out on nondenaturing slab gels of 12.5% polyacrylamide containing ampholines (pH 3.0 to 9; Pharmacia). A total of 100 μg of proteins were run at 600 V/h for 45 minutes. The pH gradient was calibrated by using an Isoelectric Focusing Calibration Kit Broad pl (pH 3-10) (GE Healthcare, UK Limited). The gels were fixed in 20% trichloroacetic acid–30% methanol–3.5% sulphosalicylic acid and the migrated proteins were stained with silver, according to the manufacturer’s recommendations using the GE Health Care standard focusing equipment. The experiment was repeated six times and the analysis of each sample was made by means of a GENE GENIUS gel system (Syngene Synoptics Ltd, Cambridge, UK).

**RESULTS AND DISCUSSION**

**PROTEIN EXPRESSION.**

pVVGJΦ and pVCTXΦ were apparent in protein profiles of whole cell extracts of VGJΦ-infected but not in VGJΦ-uninfected cultures (Fig.1 A and B: lane 3 versus lane 2). The SDS-PAGE estimated molecular weight was 12.7 kDa and 16 kDa, respectively (Fig.1 A and B: lane 3). These sizes match well with that of the formerly known orf112 gene
product as determined by mass spectrometry (12.72 kDa) (9) and recombinant rstB-his (16.8 kDa).

**ISOLATION AND PURIFICATION OF PV\(^{VGJ\Phi}\) PROTEIN.**

Figure 1 shows the SDS-PAGE monitoring of the purification process of pV\(^{VGJ\Phi}\) and pV\(^{CTX\Phi}\) proteins. Fractions obtained after purification process (lane 10 (A) and lane 7 (B)) showed a single band with a molecular mass of 12.7 kDa and 16 kDa, respectively. The proteins were purified to more than 90% homogeneity. Based on the protocols previously reported (10) it was possible obtain enough amounts of bioactive proteins for their biochemical characterization.

**BIOCHEMICAL CHARACTERIZATION OF THE PV\(^{VGJ\Phi}\) PURIFIED PROTEIN.**

**ISOELECTRIC FOCUSING.**

Isoelectric focusing of the protein gave a strong main band with a pl estimated at 6.82 for pV\(^{VGJ\Phi}\) and 6.47 for pV\(^{CTX\Phi}\) protein (not shown). This corresponds well with the NTI vector suite 6 program (InforMax, Inc.) *in silico* predicted value of 6.52 and 6.46, respectively.

**MOLECULAR WEIGHT DETERMINATION OF PV\(^{VGJ\Phi}\) PROTEIN BY HPLC-SEC.**

The purified proteins preparation was analyzed by HPLC-SEC on a Superdex 200 HR 10/30 column to determine the oligomeric state of both proteins. Fig. 2A depicts the chromatogram obtained for pV\(^{VGJ\Phi}\) where one major peak eluted from the column at an elution volume of 18.52 mL. The calculated molecular weight of this protein species was 27 kDa. This value corresponds closely with the expected molecular weight of the dimeric form of the predicted gene V\(^{VGJ\Phi}\) product (25.44 kDa). Also, other minor peak was detected at 17.6 mL with an estimated molecular weight of 53.5 kDa, which is close to that of 50.88 kDa bioinformatically predicted for a tetrameric architecture of pV\(^{VGJ\Phi}\). On the other hand in Fig. 2B can be seen the corresponding chromatogram for pV\(^{CTX\Phi}\) protein, showing a major peak eluted at 17.34 mL. The calculated molecular weight of this protein species was 65 kDa. This value matches with a homotetrameric state (64 kDa) for this protein.

These data suggest that the pV\(^{VGJ\Phi}\) native protein occurs mostly as a homo-dimer and in minor proportion as a tetramer, while pV\(^{CTX\Phi}\) native protein occurs mostly as a homotetramer. Dimers are the more usual form reported for bacteriophage SSBs, such as gene V protein from bacteriophage f1 (11), the Pf3 ssDBP, (encoded by filamentous phages hosted by *Pseudomonas*) (12), and the product of Gene 2.5 from Bacteriophage T7 (13). Also the Arc repressor from *Salmonella* (14) and Dmu encoded by *Deinococcus murrayi* (15) share the homodimeric architecture. Tetrameric structures are also reported as usually functional in bacteria (16-19) but less frequently among bacteriophages.

**FUNCTIONAL ASSAY.**

The specificity of binding of these proteins for ssDNA or dsDNA and the extent of binding achieved with increased amounts of proteins and after different reaction times were also investigated.

Under the experimental conditions tested, the purified pV\(^{VGJ\Phi}\) protein bound ssDNA of V\(^{GJ\Phi}\) starting at an effective concentration of 0.03 µg·µl\(^{-1}\) up to apparent saturation at 0.5 µg µl\(^{-1}\). As shown in Fig. 3 A, lanes 2 to 9, increased amounts of the lowest mobility ssDNA- pV\(^{VGJ\Phi}\) complex appeared with increasing pV\(^{VGJ\Phi}\) protein concentrations. At the
highest protein amount tested (23 µg), virtually all of the VGJΦ genomic ssDNA was shifted to the lowest mobility complex (Fig. 3A, lane 9). The protein - DNA complexes were also formed with the unrelated single stranded DNA from pBlueScript SK (+) as depicted in Fig 3 B, but not with the dsVGJΦ replicative form or with the dsDNA of plasmid pUC19 (Fig. 3 C). These results indicate that pVVGJΦ has general affinity for ssDNA. However, the purified pVCTXΦ protein bound ssDNA as well as dsDNA of VGJΦ, starting at a minor effective concentration, from 0.015 µg·µl⁻¹ up to apparent saturation at 0.5 µg µl⁻¹. As shown in Fig. 3 D and E, increased amounts of the lowest mobility ssDNA-pVCTXΦ and dsDNA-pVCTXΦ complexes appeared with increasing pVCTXΦ protein concentrations. At the highest protein amount tested (20 µg), virtually all of the VGJΦ genomic ssDNA (Fig. 3D, lane 8) and replicative dsDNA (Fig. 3E, lane 8) was shifted to the lowest mobility complex. As can be deduced from this picture, the pVCTXΦ exhibited higher affinity for the replicative form dsDNA than ssDNA of VGJΦ. No retardation was observed with protein preparations from negative controls or when the DNA-protein mixture was inactivated with 1:1 (vol/vol) phenol-chloroform indicating that DNA retardation in gels occurs due to non-covalent binding and requires the presence and activity of these proteins. Since pVCTXΦ has affinity for both ss and dsDNA, also was investigated whether pVCTXΦ has more affinity for the phage ssDNA than for a non related dsDNA. A DNA-binding competition experiment in which the protein was incubated with a constant amount of ssDNA of VGJΦ phage and increasing amounts of calf thymus DNA was performed (Fig.3F). The ssDNA of VGJΦ was retarded by pVCTXΦ even in the presence of 500 and 1,000 ng of calf thymus dsDNA (Fig. 3F, lanes 3 and 4). These results indicate that pVCTXΦ protein has more affinity for the phage ssDNA than for a non-phage-related dsDNA. Until we know more, pVCTXΦ is the first protein of a filamentous phage which shows affinity for both ss- and dsDNA, at least in vitro. It is possible that pVCTXΦ needs another protein from the host or the phage itself to recognize the ssDNA in a specific manner, or perhaps the affinity of pVCTXΦ for both ss-and dsDNA is an intrinsic property of the protein, which is needed on one hand for binding to genomic ssDNA during the rolling circle replication of the phage and on the other hand for holding the hairpin dsDNA secondary structure formed by the phage genome that functions as the site for integration into the bacterial chromosome (20). This hairpin structure is used by XerCD recombinases as a substrate for recombining the phage genome with the bacterial chromosomal dif site (20), and pVCTXΦ may act jointly with XerCD to achieve integration. This could explain the requirement of pVCTXΦ for the integration of CTXΦ, described before (21).

The influence of binding reaction times from 0 to 20 min. on protein-DNA complexes formation was examined. As per our EMSA results, pVVGJΦ protein was found to almost instantly bind the VGJΦ-ssDNA (Fig. 4A). The same behaviour was seen for pVCTXΦ with both ss and dsDNA of VGJΦ (Fig. 4B) Once the fully saturated complexes were formed, they remained apparently unchanged upon further prolonged incubations of up to 20 min. Strikingly, these complexes were formed faster than similar complexes in other species. M 13 mp18 ssDNA with SSB proteins such as Balf2ΔC encoded by Epstein-Barr virus and ICP8ΔC encoded by herpes simplex virus I, required 3 and 5 min for assembly, respectively (22).

CONCLUSIONS.

This paper describes the partial characterization of the proteins pVVGJΦ and pVCTXΦ, encoded by gV the phages VGJΦ and CTXΦ, respectively in Vibrio cholerae. According to the results obtained in their biochemical characterization, it is concluded that pVVGJΦ is a ssDNA binding proteins with general affinity for ssDNA. However pVCTXΦ is a DNA binding protein, with affinity for both ss and dsDNA pVCTXΦ, with apparently more affinity for the phage dsDNA of VGJΦ and minor affinity for a non-
phage-related dsDNA. Also it was shown for the first time that the same as the majority of the ssDNA binding proteins, $pV^{VgJ\phi}$ forms homodimers while $pV^{Ctx\phi}$ occurs fundamentally as homotetramers.
FIG. 1. SDS-PAGE monitoring of purification process. (A) Isolation and purification of pV^VGJΦ. Lane 1, Broad Range Protein Molecular Weight Marker (Promega, USA); lane 2, cell extract of VGJΦ uninfected *V. cholerae* 569B; lane 3, cell extract of VGJΦ-infected *V. cholerae* 569B; lane 4, soluble fraction of the sonicate; lane 5, insoluble fraction of the sonicate; lane 6, precipitate at 30% (NH₄)₂SO₄; lane 7, supernatant at 30% (NH₄)₂SO₄; lane 8, precipitate at 50% (NH₄)₂SO₄; lane 9, supernatant at 50% (NH₄)₂SO₄; lane 10, pV^VGJΦ electroeluted from preparative SDS-PAGE. (B) Isolation and purification of pV^CTXΦ. Lane 1, broad-range protein molecular mass markers (Promega); lane 2, cell extract of uninduced cultures; lane 3, cell extract of expression-induced cultures; lane 4, soluble fraction of the sonicate from expression-induced cultures; lane 5, insoluble fraction of the sonicate from expression-induced cultures; lane 6, soluble fraction of the 8 M urea extract; lane 7, pV^CTXΦ electroeluted from the column.
FIG. 2. Characterization of the purified proteins: $\text{pV}^{\text{VGJ}}$ (A) and $\text{pV}^{\text{CTX}}$ (B). Chromatogram $\text{DO}_{280}(\text{UA})$ versus retention time, obtained after HPLC-size exclusion chromatography of 100 $\mu$g of the purified proteins through a Superdex 200 HR 10/30 column. $\text{DO}_{280}(\text{UA})$, optical density reads at 280 nm wavelength given in arbitrary units.
FIG 3. Electrophoretic mobility shift assays. (A) Protein – DNA binding in response to increasing doses of pV^VGJΦ against 0.5 µg of VGJΦ-ssDNA. Lanes 1 – 9, ssDNA plus 0, 0.6, 1.25, 2.50, 5.00, 10.0, 16.2, 20.0 and 23.0 µg of pV^VGJΦ, respectively; lane 10, the same as 8, but extracted with phenol/chloroform. (B) Specificity of binding of pV^VGJΦ (16.0 µg) against unrelated single stranded DNA of pBlueScript SK (+). Lane 1, control of pBlueScript SK (+) ssDNA; lane 2, same as 1 plus 16.0 µg of pV^VGJΦ. (C) Specificity of binding of pV^VGJΦ (16.0 µg) against double stranded linear DNA. lane 1, control of dsDNA (linearized replicative form of VGJΦ); lane 2, same as 1 plus 16.0 µg of pV^VGJΦ; lane 3, control dsDNA of linearized pUC19; lane 4, same as 3 plus 16.0 µg of pV^VGJΦ. (D) Binding of pV^CTXΦ to genomic ssDNA of VGJΦ. Lane 1, control of 500 ng of ssDNA of VGJΦ; lane 2, same as for lane 1 plus 20 g of pV^CTXΦ treated with phenol-chloroform; lanes 3 to 8, same as for lane 1 plus 0.62, 1.25, 2.50, 5.00, 10.0, and 20.0 µg of pV^CTXΦ, respectively; lane 9, linearized replicative-form dsDNA of VGJΦ; lane 10, same as for lane 9 plus 20.0 µg of pV^CTXΦ; lane 11, linearized pUC19; lane 12, same as
for lane 11 plus 20.0 µg of pV^CTX\Phi_. (E) Binding of pV^CTX\Phi_ to dsDNA (linearized replicative form of VGJ\Phi). Lane 1, control of 500 ng of dsDNA of VGJ\Phi; lanes 2 to 7 same as for lane 1 plus 0.62, 1.25, 2.50, 5.00, 10.0, and 20.0 µg of pV^CTX\Phi_, respectively. (F) lane 2 plus 100, 200, 300, 400, and 500 mM NaCl. (C) DNA-binding competition by pV^CTX\Phi_. Lane 1, 400 ng of genomic ssDNA of VGJ\Phi (control); lane 2, same as for lane 1 plus 15.6 µg of pV^CTX\Phi_; lanes 3 and 4, same as for lane 2 plus 500 and 1,000 ng of sheared calf thymus dsDNA, respectively; lane 5, 500 ng of calf thymus DNA (control).
FIG 4. Evaluation of reaction times in the protein DNA complexes formation. Panel A. pV^VGJ^Φ protein Lane 1, 0.5 µg of control VGJ^Φ-ssDNA, lanes 2-6, same as lane 1 plus 5.0 µg of pV^VGJ^Φ protein incubated for binding reaction times of 0, 5, 10 15 and 20 min. (B) pV^CTX^Φ protein Lane 1, 0.5 µg of control VGJ^Φ-ssDNA, lanes 2-5, same as lane 1 plus 5.0 µg of pV^CTX^Φ protein incubated for binding reaction times of 0, 5, 10 and 20 min. (C) pV^CTX^Φ protein Lane 1, 0.5 µg of control VGJ^Φ-dsDNA, lanes 2-5, same as lane 1 plus 5.0 µg of pV^CTX^Φ protein incubated for binding reaction times of 0, 5, 10 and 20 min.
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