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Supramolecular Complexes of the *Agrobacterium tumefaciens* Virulence Protein VirE2

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Abstract—Virulence protein VirE2 from *Agrobacterium tumefaciens* is involved in plant infection by transferring a fragment of agrobacterial Ti plasmid ssT-DNA in complex with VirE2-VirD2 proteins into the plant cell nucleus. The VirE2 protein interactions with ssDNA and formation of VirE2 protein complexes *in vitro* and *in silico* have been studied. Using dynamic light scattering we found that purified recombinant protein VirE2 exists in buffer solution in the form of complexes of 2-4 protein molecules of 12-18 nm size. We used computer methods to design models of complexes consisting of two and four individual VirE2 proteins, and their dimensions were estimated. Dimensions of VirE2 complexes with ssDNA (550 and 700 nucleotide residues) were determined using transmission electron microscopy and dynamic light scattering. We found that *in vitro*, upon interaction with ssDNA recombinant protein, VirE2 is able to alter conformation of the latter by shortening the initial length of the ssDNA.

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Under natural conditions bacteria of the genus *Agrobacterium* are able to transfer ssT-DNA complexed with VirE2 and VirD2 proteins into plant cells and incorporate them into the host cell chromosomes [1, 2]. Any genes localized between right and left T-DNA borders can be transferred within the latter. Expression of transferred genes in the host cell changes its properties, which has already become widely used in agricultural biotechnology for creation of transgenic plants, and it opens the perspective of biomedical applications in gene therapy of animal cells, because T-DNA transfer into animal cells has already been shown [3].

The ssT-DNA–VirD2 complex and VirE2 protein are transferred into plant cell independently, but VirE2 export depends on the presence of VirE1 protein [4, 5]. In this case transfer of the VirE2–VirE1 complex is carried out via a VirB-independent channel [6]. VirE2 protein is one of the major virulence proteins of *A. tumefaciens*; it exhibits the ability for nonspecific and highly cooperative binding to ssDNA, its protection against plant nucleases, and it is involved in transport from membrane to the cell nucleus [7-9]. In addition, the ability of VirE2 protein to interact with planar lipid membrane was found, which increases its electric conductivity and forms a pore [10, 11]. However, it is still unknown how T-DNA is transferred through host-cell membrane with involvement of VirE2 protein. In particular, it is not clear in what form VirE2 protein exists after dissociation of the VirE2–VirE1 complex.

The goal of this work was to investigate the supramolecular complexes of VirE2 protein with VirE2–ssDNA *in vitro* and in *in silico*.

MATERIALS AND METHODS

Strains, plasmids, media. *Escherichia coli* XL1-Blue with plasmid pQE-31 (Promega, USA) grown on Luria–Bertani medium were used.

Isolation and purification of recombinant protein VirE2 was carried out as described previously [9]. The isolation procedure was as follows: recombinant protein

Abbreviations: a.a., amino acid residue; bp, base pair; DLS, dynamic light scattering; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; T-DNA, transfer DNA; TEM, transmission electron microscopy.

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VirE2 with six consecutive histidine residues was isolated from *E. coli* XL1-Blue strain containing pQE31 plasmid with the *vir*E2 gene. After centrifugation, the *E. coli* cells were suspended in buffer A (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM imidazole, 1 mM PMSF, pH 8.0) and sonicated. After centrifugation, the supernatant was applied on a Ni-NTA (nickel-nitrilotriacetic acid) column (Amersham Pharmacia Biotech., GB). Nonspecifically bound proteins were washed off by four volumes buffer B (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0). VirE2 was eluted in buffer C (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0) and desalted on a Sephadex G-25 column. Electrophoresis of protein preparations was carried out in 12.5% SDS-polyacrylamide gel as described in [12]. The protein was additionally purified by fractionation in tubes with Princeton Separation membrane filters (USA).

Protein size determination by dynamic light scattering (DLS). Particle size distribution was studied in a liquid media Zetasizer Nano series ZEN 3500 (Malvern Instrument LTD, GB) with the Dispersion Technology Software 4.2 program (Malvern Instrument LTD). Hydrodynamic diameter of the protein was measured in 50 mM NaH_2PO_4, 50 mM NaCl buffer, pH 8.0. Concentration of VirE2 protein defined by the Bradford technique was 180 μg/ml. BSA at concentrations 100, 250, and 500 μg/ml was used as standard.

Isolation of ssDNA. Preparation of *A. tumefaciens* dsDNA was obtained using PCR technique. Plasmids pQE-GFP [13] and pBi2E were used as templates [14].

To obtain the PCR fragment (700 bp) of the *gfp* gene from plasmid pQE-GFP, the following primers synthesized by the Syntol (Russia) were used: GFP(+) 5′-tgt cag tgg aga ggg tga agg-3′; GFP(–) 5′-aca ggg cca tcg cca att gga-3′. PCR was carried out in the following temperature regime: 5 min at 95°C; 35 cycles: 1 min at 94°C, 1 min at 60°C, 1 min at 72°C, 2 min at 72°C, cooling at 4°C.

To obtain the 550 bp PCR fragment of the proline dehydrogenase gene, the direct (5′-aac aaa ctg gat ccg gcg atc ttac-3′) and inverse (5′-gag atg ttg gtc tag att tgg cagc-3′) primers (Syntol) were used under the following conditions: preliminary DNA denaturing at 95°C for 3 min, then cycle – 1 min at 95°C, 1 min at 58°C, 1 min at 72°C (35 cycles). When the last cycle was over, final chain completion was carried out at 72°C for 3 min. Then dsDNA was converted to ssDNA by denaturing at 95°C for 5 min and quick subsequent cooling on an ice-water bath.

Transmission electron microscopy (TEM) of DNA–protein complex. To form ssDNA–protein complexes, 20 μl of mixture consisting of 0.2 μg of ssDNA of the *gfp* gene PCR fragment and 1.8 μg (or 5.4 μg) of VirE2 virulence protein was placed in 0.02 M Tris-HCl, 5 mM $MgCl_2$ buffer, pH 7.2. Complex was formed by incubation for 20 min at 4°C, after which the preparation was applied on Parafilm, and a grid (300 mesh) with Formvar support was placed onto the drop and left for 15 min at room temperature. Then the grid was washed three times in water (miliQ). The preparation was contrasted with 2% aqueous uranyl acetate solution for 1 min, and the grid was again washed three times in water and air-dried. Preparations were examined in a Libra 120 electron microscope (Carl Zeiss, Germany).

Computer analysis of VirE2 protein. To design complex structures of VirE2 and VirE2-VirE1 proteins, the GRAMM-X program (Protein Docking WebServer v.1.2.0, http://vakser.bioinformatics.ku.edu/resources/gramm/grammx) was used along with the Hex program for modeling protein–protein interactions [15] available on server http://hexserver.loria.fr.

RESULTS AND DISCUSSION

Measurement of VirE2 protein size by DLS. BSA, similar in molecular mass to VirE2, was measured as a control. BSA in solution was mainly present as 8 nm particles (93% of all particles), while 7% of all particles were aggregates of 68 nm (table).

For computer protein sizing, the BSA model (UniProt ID P02769) based on the human serum albumin structure 2BXH (http://swissmodel.expasy.org/repository) was used, because there is no X-ray model for BSA. BSA dimensions were estimated in the Swiss-PDB

Dimensions of complexes formed by BSA and VirE2 proteins and by VirE2 protein with ssDNA

Variant	Mean complex diameter, nm	Complex portion in total population, %
BSA	8 68	93 7
VirE2	12 115 2800	51 47 2
VirE2 (double freezing-thawing)	12 115 2800	28 70 2
VirE2 after 16-h incubation at 4°C	18 151 3000	37 60 3
dsDNA (550 base pairs)	220 180 250	92 6 2
ssDNA (550 nucleotide residues) with VirE2 (30-min combined incubation)	140 21	94 6

Note: DNA amount was 0.2 μg, VirE2 concentration 2.0 μg.

Viewer 4.0.1 program by measuring distances between the atoms of the most remote amino acid residues (a.a.). It was shown that the BSA globule has the shape of a cone with characteristic dimensions 8 nm (Asn144-Asp387) and 7.7 nm (Ala584-Ser82), i.e. there is significant similarity in size between computer model and our measurement of BSA by DLS in solution.

The VirE2 solution after the Ni-NTA-agarose column by half (51%) consists of particles with hydrodynamic diameter of 12 nm, 47% of all particles were aggregates of mean diameter 115 nm, and just a small part of all aggregates (2%) had diameter of 2800 nm (table). After double freeze-thawing procedure the amount of aggregates increased to 70% due to lowering amount of 12 nm particles to 28%. After incubation for 16 h at 4°C, 37% of all particles were in 18 nm aggregates, 60% in 151 nm aggregates, and 3% in 3000 nm aggregates.

Computer modeling of crystallized part of VirE2 protein using the Swiss-PDB-Viewer program showed that dimensions of the protein do not exceed those of a cell $8 \times 6 \times 5$ nm (8 nm between Lys488 and Thr141, 6 nm between Pro184 and Thr297, and 5 nm between Arg225 and Lys298). Earlier we used the PROFsec (PredictProtein) program to obtain secondary structure for complete amino acid sequence of VirE2 protein [11] and comparatively analyzed VirE2 three-dimensional structure (PDB ID:3BTP) based on data of X-ray analysis [16]. At the N terminus of VirE2 3D structure (PDB ID:3BTP), among absent (in the model based on X-ray data) 111 a.a., the PROFsec program predicted the 14 a.a. helix of ~1.5 nm (estimated in the Swiss-PDB-Viewer program). No specific structures were predicted at the C terminus. On the whole, the missing 139 a.a. may provide for ~37% of the whole molecule length (mass and volume), which may increase volume dimensions of the VirE2 monomer by 10% to $9 \times 7 \times 6$ nm.

At the same time, the VirE2 dimensions within ssDNA–VirE2 complex, obtained by three-dimensional reconstruction on the basis of data of electron microscopy, was $8.1 \times 5.2 \times 4.3$ nm [17]. This means that the contribution of missing protein fragments to estimation of protein real dimensions is not so significant. Nevertheless, below we will give the rounded computer values for dimensions of VirE2 complexes with account for missing amino acid residues.

Thus, the single VirE2 molecule obtained by X-ray analysis taking into account the absent in the model 139 a.a., can be described in C and N domains by a cell of $9 \times 7 \times 6$ nm.

Since the size of VirE2 protein, measured by three-dimensional reconstruction techniques based on electron microscopy data [17] and our computer reconstruction based on X-ray analysis data, was significantly lower than the VirE2 diameter measured by DLS, the question arises whether VirE2 protein exists in solution as a single molecule or in complexes. It is known that the VirE1 protein forms a complex with VirE2 in an agrobacterial cell due to interaction of the latter at binding sites that simultaneously serve for contact with ssT-DNA upon T-complex formation in the plant cell cytoplasm [16, 17]. In the absence of VirE1 protein, VirE2 is inclined to aggregation [18]. The VirE2 size, measured by DLS in solution, was 12-18 nm (table). It can be supposed that the VirE2 protein after column purification exists as a complex of two or more proteins.

Since aggregation may influence VirE2 interaction with membrane and ssDNA as well as the self-assembly of VirE2 protein, the problem of VirE2 complex dimensions will be considered below based on computer models.

Estimation of the size of complex of two VirE2 proteins constructed using the Hex program. Dimensions of a complex of two VirE2 proteins are shown in Fig. 1 and are equal to 7 nm (between Thr141 and Tyr414 of protein shown in white), 8 nm (between Glu269 of the protein shown in white and Arg435 of the protein shown in gray) (Fig. 1a), and 5 nm (between Glu239 and Gln174 of the protein shown in white) (Fig. 1b). With account of amino acid residues absent in the model structure at the C and N termini for each protein, dimensions of the complex of two VirE2 proteins will not exceed cell dimensions $10 \times 8 \times 6$ nm. In a complex of two symmetrically arranged VirE2 proteins, built using the Hex program, the region of contact between proteins is ~30 a.a. Leu182-Tyr194 and Gln216-Arg234 of the first protein C domain contact Glu463-Val480 and Lys507-Ala517 of the second VirE2 protein N domain. Since the complex of two VirE2 proteins is symmetrical, the analogous amino acid residues contact at the other terminus.

Estimation of the size of a complex of two VirE2 proteins built using the GRAMM-X program. Dimensions of such complex built using the GRAMM-X program taking into account the amino acid residues absent in the model [16] at the C and N termini are $9 \times 8 \times 6$ nm. The region of contact between two VirE2 proteins in one of two contact spots is about 10 a.a. in each protein: Asp233-Ile246 of the first protein C domain contact Glu500-Leu516 of the second VirE2 protein N domain. Since complexes of two proteins with two contact spots built using GRAMM-X and Hex programs are symmetrical, the analogous amino acids contact in the other contact the spot.

Thus, models of complexes of two VirE2 proteins with two contact spots according to estimations of two programs (GRAMM-X and Hex) have dimensions $(9\text{-}10) \times 8 \times 6$ nm, which is less than VirE2 protein dimensions measured by the DLS technique (table).

Among models built using the GRAMM-X program, there is the complex of two VirE2 proteins with a single contact spot. The distance of 14 nm was measured between Thr141 of protein *1* and Thr141 of protein *2* in the model [16]. The distance of 6 nm was measured between Pro184 and Thr297 of protein *1*. Distance of 5 nm was measured between Arg225 and Lys298 of pro-

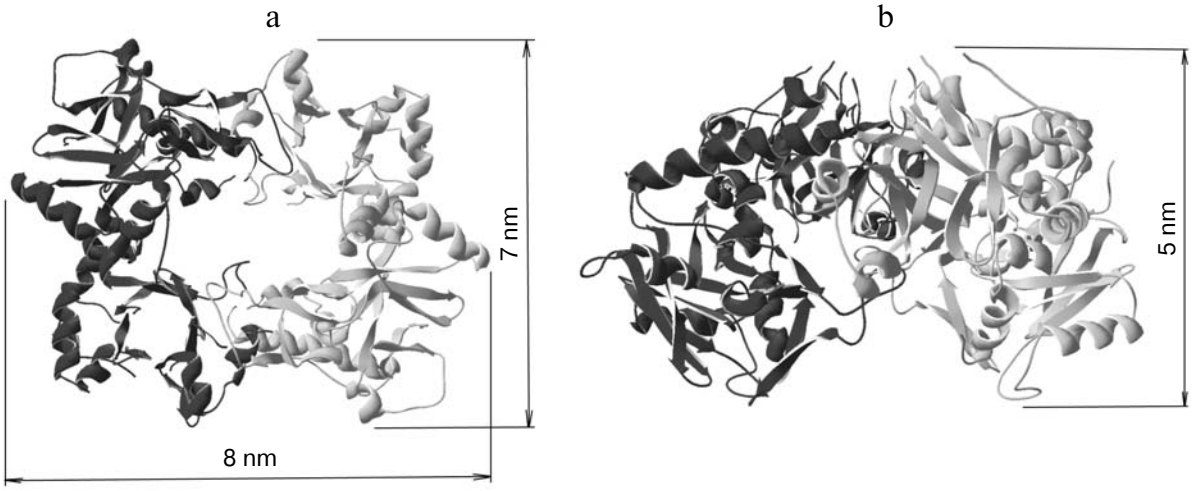

Fig. 1. Dimensions of a complex of two VirE2 proteins obtained on the basis of three-dimensional structure of a single VirE2 protein (PDB ID:3BTP) using the Hex program: a) view from above; b) side view. With account of amino acid residues absent in the model structure at the C and N termini, for each protein, complex dimensions will not exceed those of a cell 10 × 8 × 6 nm.

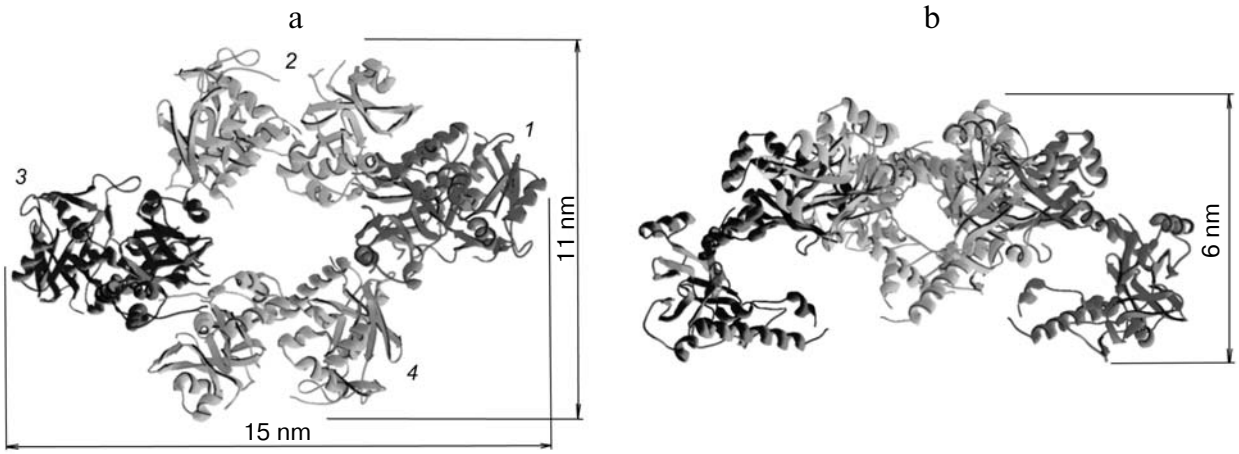

Fig. 2. Complex of four VirE2 built using the GRAMM-X program on the basis of three-dimensional structure of a single VirE2 protein (PDB ID:3BTP): a) view from above; b) side view. With accounting for amino acid residues absent in model structure at C and N termini, for each protein, complex dimensions will not exceed those of a cell 17 × 13 × 8 nm.

tein *2* in the model [16]. With accounting for the fact that, according to our modeling data, in the absent N terminus of the VirE2 3D structure (PDB ID:3BTP) 111 a.a. can exist in the form of α-helix, then the complex size may be equal to 15 × 7 × 6 nm. Thus, the size of the complex of two VirE2 proteins with a single contact spot, to a greater extent corresponds to data of the VirE2 hydrodynamic diameter measurements by DLS.

Sizing the complex of four VirE2 proteins built using the GRAMM-X program. It was supposed earlier that a membrane pore complex can be formed by four subunits of VirE2 protein [19].

Formation of VirE2 tetramer within the ssDNA–VirE2 complex was confirmed by TEM [17]. A complex of four VirE2 proteins built using the GRAMM-X program on the basis of X-ray data for the VirE2–VirE1 protein complex [16] is a closed-type ring-like roll the dimensions of which are shown in Fig. 2. The distance 15 nm is measured between Lys298 of protein *3* and Lys298 of protein *1*, distance 11 nm is measured between Glu232 of protein *2* and Arg229 of protein *4*, and distance 6 nm is measured between Lys189 and Glu418 of protein *1*. A complex of four VirE2 proteins built using the GRAMM-X program on the basis of X-ray structural analysis data of VirE2–VirE1 protein complex may look like the following: Glu173-Gln193 of protein *1* N domain contact Pro430-Asp448 of protein *2* domain C; Ala129-Glu144 of protein *2* domain N contact Arg503-Ala517 of

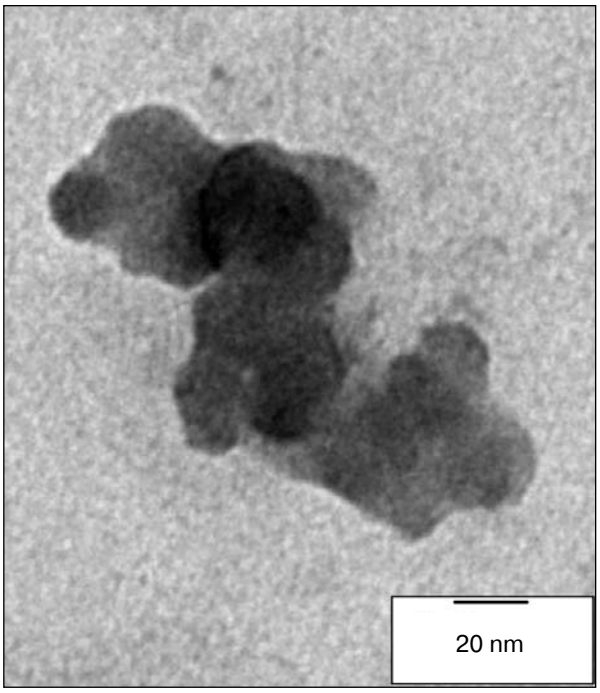

Fig. 3. TEM of DNA–protein complex consisting of ssDNA (PCR fragment of *gfp* gene) and VirE2 protein taken in the ratio 1 : 10. Contrasting by 2% uranyl acetate.

protein *3* domain C; Gln356-Arg365 and Arg435-Ala439 of protein *3* domain C contact Ser432-Gln450 of protein *4* domain C; Lys133-Thr141 of protein *4* domain C contact Ser468-Asp479 and Met505-Leu515 of protein *1* domain C.

Dimensions of the complex of four VirE2 proteins taking into account amino acid residues absent in model structure at C and N termini will not exceed 17 × 13 × 8 nm.

Thus, dimensions of model complexes of two VirE2 proteins with a single contact spot and of four VirE2 proteins are close to the size of particles of VirE2 protein measured by DLS (table).

Formation of VirE2 proteins with ssDNA. The VirE2 protein belongs to the SSB protein family interacting with single-stranded DNA, which nonspecifically binds to ssDNA [9]. The ssT-DNA–VirE2–VirD2 complex (T complex) formed *in vitro* is a structure resembling "a telephone cord-like coiled structure" which prevents T-DNA rolling up to globular form [16, 17, 20-22]. The mean size of the 550 bp long dsDNA measured by DLS was 220 nm in the case of ssDNA length in the range from 180 to 250 nm (table). Upon the interaction of VirE2 protein (2 μg) with 550 bp long ssDNA (0.2 μg), in 30 min after the beginning of combined incubation 140 nm complexes were registered (94%) and particles of 17-30 nm made up 6% (table). It is seen that the presence of VirE2 protein results in reduction by one third (36%) of the initial ssDNA length, which may be indicative of formation of DNA–protein complexes.

Measurement of VirE2 supramolecular complexes by TEM. To form VirE2–ssDNA complex *in vitro*, VirE2/ssDNA in a ratio 10 : 1 and 30 : 1 in buffer D were used. If the length of a single nucleotide is 0.34 nm, then ssDNA of 700 nucleotides should be 238 nm long. At the VirE2/ssDNA ratio 10 : 1 the measured length of T complex was 140 nm (59% of initial ssDNA length) (Fig. 3). At the VirE2/ssDNA ratio 30 : 1 the complex length was 146 nm, or 61% of the initial ssDNA length (700 nucleotides) measured by TEM. It was noted in [17] that in the case of ssDNA complex formation with VirE2, ssDNA length became seven times shorter, whereas no such significant differences were observed in our studies. Evidently this could be due to differences in the length and sequence of used ssDNA. In our experiments the width of VirE2–ssDNA complex varied from 15 to 25 nm and on the average was 15.8 nm (50 measurements). According to data of Abu-Arish et al. [17], the diameter of the complex was 15.4 nm, i.e. the width of ssDNA–VirE2 complexes measured by us and in [17] by TEM correlated well.

Thus, recombinant protein VirE2 in buffer solution can exist in complexes of two to four proteins and form DNA–protein complex upon interaction with ssDNA, thus shortening the ssDNA.

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