Purification, characterization, assembly and crystallization of assembled alfalfa mosaic virus coat protein expressed in *Escherichia coli*

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The coat protein of alfalfa mosaic virus (AMV) was cloned and expressed in *Escherichia coli* as a fusion protein containing a 37 amino acid extension with a (His)_6 region for affinity purification. About half of the expressed recombinant coat protein (rCP) was soluble upon extraction and half was insoluble in inclusion bodies. Western blot analysis confirmed the identity of the rCP and protoplast infectivity assays indicated that the rCP was biologically active in an early event of AMV infection, called genome activation. The rCP assembled into *T*=1 empty icosahedral particles, as described previously for native coat protein. Empty particles formed hexagonal crystals that diffracted X-rays to 5.5 Å resolution. The crystals of trypsin-treated particles of rCP appear to be isomorphous with crystals of trypsin-treated particles of native coat protein. Spherical particles containing RNA assembled when the rCP was combined with *in vitro* transcripts of AMV RNA4, the smallest naturally encapsidated AMV RNA. Bacilliform particles that resembled native virions assembled when the rCP was combined with transcripts of RNA1, the largest genomic RNA.

Introduction

The coat proteins (CPs) of many plant viruses are multifunctional and required throughout infection, in addition to being required for particle assembly. For example, the CP of some viruses is directly or indirectly involved in the accumulation of viral plus-strand RNA (Marsh et al., 1991; Van der Kuyl et al., 1991b; Chapman et al., 1992; Van der Vossen et al., 1994; De Graaff et al., 1995). Many CPs are also required for the virus infection to spread from cell-to-cell (Van der Kuyl et al., 1991a; Van der Vossen et al., 1994; Dolja et al., 1994; Chapman et al., 1992) and/or for systemic spread throughout the plant (Sacher & Aihquist, 1989; Allison et al., 1990; Dolja et al., 1994). The CP of alfalfa mosaic virus (AMV) is involved in all of these functions, as well as being required at the start of infection (Bol et al., 1971; Quadt et al., 1991). Mutational analysis of AMV CP has suggested that separate regions of the protein are important for different functions (Van der Vossen et al., 1994). For example, the N terminus of the CP is involved in an early event of infection, called genome activation, which requires binding of the N terminus to the 3' untranslated region of AMV RNAs (Baer et al., 1994).

The C terminus is required for virion assembly (Van der Kuyl et al., 1991a; Van der Vossen et al., 1994). Thus, regions of the CP involved in different functions may be mutated separately. However, mutations in regions involved in early functions, such as genome activation, which affects replication, will also affect later functions, such as assembly. Therefore, to study the effects of CP mutations on assembly and structure, we adapted an *in vitro* assembly system described by Bukuyama et al. (1981) for use with AMV CP expressed in *Escherichia coli*. Thus, AMV assembly can be uncoupled from replication.

AMV particles are bacilliform which is unique among plant viruses. There is evidence that some viruses in the related *ilarvirus* genus may have bacilliform particles (Halk, 1981); however, these virus particles do not appear to be as stable as those of AMV. RNA–protein interactions primarily stabilize AMV particles. If the N terminus of CP in particles is removed by mild trypsin treatment, the particles lose their bacilliform shape and became spherical (Bol et al., 1974). If the particles are completely disrupted by salt, reassociation of the bacilliform particles is not possible *in vitro* (Hull, 1970). This is in contrast to the reversible dissociation of viruses, such as cowpea chlorotic mottle virus, which will reassocicate *in vitro* (Bangcroft & Hiebert, 1967; Zhao et al., 1995). In the absence of RNA, AMV CP dimers self-associate to form empty *T*=1 icosahedral particles, which have been crystallized and analysed by X-ray
diffraction (Fukuyama et al., 1983). In the presence of various RNA or DNA molecules, the CP aggregates into spherical or ovoid particles or into tubular particles with lengths characteristic of the nucleic acid (Hull, 1970; Driedonks et al., 1977, 1978).

In this paper we describe the assembly of icosahedral T = 1 particles by recombinant AMV CP expressed in E. coli. Particles containing RNA4, the subgenomic messenger for CP, or RNA1, the largest genomic RNA, assembled when RNA transcripts were included in the in vitro assembly reaction.

**Methods**

**Cloning of the AMV CP gene for expression in E. coli.** The CP gene was cloned by PCR using the previously described first strand primer, 5' GGGGATCCCTAGGGCCCGAG 3' (Loesch-Fries et al., 1985) and second strand primer, 5' TCGAGATCTGCAGTCATGAGTTC- TTC 3' with pSP65A4 as the template (Loesch-Fries et al., 1985) so that a PstI restriction site (indicated by double underlining) was placed just upstream of the CP ATG codon (indicated by single underlining). The PCR product containing the CP gene was cloned into the pGEM-T vector (Promega). A PstI fragment, containing the CP ORF and the 3' untranslated region from RNA4 cDNA, was ligated into the pTrcHisB plasmid (Invitrogen), which had been linearized by digestion with PstI to create pTHB-CP (Fig. 1). The recombinant coat protein (rCP) produced from this plasmid contained 37 non-viral amino acids at the translation initiation site for the fusion protein; (His), is a polyhistidine cleavage site; AMV CP is the alfalfa mosaic virus coat protein gene; term is the transcription termination sequence from the E. coli rrnB gene.

**Expression and purification of AMV recombinant CP.** For expression of rCP, a single transformed bacterial colony (Top10, Invitrogen) was grown overnight in SOB medium (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5 mM-KCl, 10 mM-MgCl₂) (Sambrook et al., 1989). The cells were then diluted 1:100 in SOB medium containing 75 µg/ml ampicillin and grown for 3-4 h at 24°C with vigorous shaking to an OD₆₀₀ of 0-3. Protein expression was induced by adding IPTG to a final concentration of 1 mM and incubation was continued for 4 h at 24°C.

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**Preparation, inoculation and immunoassays of protoplasts.** Protoplasts were isolated from axenic tobacco plants (Nicotiana tabacum var. Xanthi-nr) and inoculated with AMV genomic RNAs (RNA1, 2, 3) alone or in combination with AMV rCP or native CP (isolated according to Kruiseman et al., 1971) using a polyethylene glycol method (Loesch-Fries et al., 1985; Samac et al., 1983). The protoplasts were collected 24 h after inoculation and assayed by immunofluorescence using polyclonal antibodies to AMV CP to determine the percentage infected (Loesch-Fries & Hall, 1980). The immunofluorescence assay detected only infected protoplasts in which AMV CP had accumulated during infection. The sensitivity of the assay was below that required to detect rCP from the inoculum.

**Transcription and isolation of virus RNA.** Capped transcripts of RNA4 (898 nt) were synthesized from pSP65A4 templates using SP6 polymerase (Epicenter) as described (Loesch-Fries et al., 1985). An aliquot of the transcription reaction was used for the inoculation of protoplasts without purification of the transcripts. Capped transcripts of RNA1 (3640 nt) were synthesized using T7 polymerase and a full-length cDNA of RNA1. A mixture of AMV genomic RNAs 1, 2 and 3 was isolated from native virions as previously described (Loesch-Fries et al., 1985).

**Assembly of empty particles and encapsidation of AMV RNA with rCP.** Empty particles were assembled by dialysis of rCP at 4°C against (1) 20 mM-sodium pyrophosphate, pH 5.5, (2) 20 mM-sodium phosphate, pH 7.0 and (3) 50 mM-sodium pyrophosphate, pH 7.0, as described by Fukuyama et al. (1981), except the length of dialysis was extended to 2 days at each step. Encapsulation of AMV RNA4 transcripts by rCP was accomplished by extensive dialysis of a suspension of rCP and transcripts at a ratio of 450 rCP: 1 RNA (w/w) in NBB at 4°C against (1) 20 mM-sodium pyrophosphate, pH 5.5 for 2 days, (2) 20 mM-sodium phosphate, pH 6.0 for 2 days and (3) 50 mM-sodium pyrophosphate, pH 6.0 for 2 days. To form particles containing AMV RNA1 transcripts, a mixture of rCP and RNA1 transcripts at a ratio of 156 rCP: 1 RNA (w/w) in NBB at 4°C against 50, 75 or 100 mM-NaCl or sodium pyrophosphate, pH 6 or 6.5 for 15 h at 4°C.

**Electron microscopy.** Assembled particles were deposited on carbon-coated grids, negatively stained with 2% uranyl acetate, and air dried. The particles were examined in a Philips EM 400 electron microscope.

**Crystallography of particles.** Empty particles of rCP were treated with trypsin at a molar ratio of 1:10000 (trypsin:rCP) at 24°C for 12-15 h prior to crystallization according to Fukuyama et al. (1981). Mild trypsin treatment removes the amino acid extension and 26 N-terminal viral amino acids from rCP (Bol et al., 1974). Empty particles and RNA4-containing particles, which were not treated with trypsin, were crystallized by dialysis of a 50 µl suspension at 12-13 mg rCP/ml against 50 ml 0.2 M-citrate buffer, pH 4.6 at 24°C as described by Fukuyama et al. (1981). To analyse the RNA in RNA4-containing particles, crystals of these particles were washed with 0.2 M-citrate buffer, pH 4.6, disrupted with 10 mM-Tris–HCl buffer pH 7.0 containing 1 mM-FDXTA, 1% SDS and 2 M-urea, and analysed by electrophoresis in a 1% agarose gel followed by staining with ethidium bromide.

**X-ray analysis of crystals.** Diffraction data were collected from the crystals of empty icosahedral particles assembled from rCP using synchrotron radiation at the A-1 station, Cornell High Energy Synchrotron Source (λ = 0.9144 Å). Experimental conditions included 0.3° oscillation angle and 150 mm crystal to detector distance with 60 s
exposure time at room temperature. Diffraction images were processed using the DENZO program (Otwinowski, 1993).

Results

Purification and characterization of rCP

The soluble fraction of rCP was purified from lysed bacteria for use in the experiments described below. The soluble fraction was about half of the total rCP in induced E. coli expressing pTHB-CP (Fig. 1). The rest of the rCP was insoluble and in inclusion bodies (data not shown). Upon purification, the yield was approximately 4 mg soluble rCP per litre of cell culture of induced E. coli. Fig. 2 shows that the rCP migrated as expected for an AMV CP fusion protein of 29 kDa containing a 37 amino acid extension at the N terminus. rCP was recognized by monoclonal antibodies to AMV CP (Fig. 2, lanes 5 and 6). Preparations of native CP contained fragmented molecules due to proteolysis during isolation as shown by the lower molecular mass bands in Fig. 2, lane 2. Purified rCP, in contrast, contained little degraded protein (Fig. 2, lanes 3 and 6).

Biological activity of rCP

The genomic RNAs of AMV require the presence of AMV CP to infect plants or protoplasts (Bol et al., 1971). To determine whether the rCP expressed in E. coli was biologically active, tobacco protoplasts were inoculated with AMV genomic RNAs (RNA1, 2, 3) and rCP. Preparations of soluble proteins from uninduced or induced E. coli containing pTHB-CP and a preparation of purified rCP were each assayed. Table 1 shows that the AMV genomic RNA preparation was not infectious without the addition of native CP or RNA4, which encodes the CP. Addition of rCP to the inoculum also

Table 1. Activity of AMV rCP in genome activation

<table>
<thead>
<tr>
<th>Inoculum*</th>
<th>Percentage infection†</th>
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<tbody>
<tr>
<td>RNA1, 2, 3</td>
<td>Experiment 1</td>
</tr>
<tr>
<td>RNA1, 2, 3</td>
<td>NT</td>
</tr>
<tr>
<td>+ uninduced soluble protein preparation from E. coli containing pTHB-CP</td>
<td>NT</td>
</tr>
<tr>
<td>RNA1, 2, 3</td>
<td>10</td>
</tr>
<tr>
<td>+ induced soluble protein preparation from E. coli expressing pTHB-CP</td>
<td>25</td>
</tr>
<tr>
<td>RNA1, 2, 3</td>
<td>NT</td>
</tr>
<tr>
<td>+ CP</td>
<td>50</td>
</tr>
<tr>
<td>RNA1, 2, 3</td>
<td>+ RNA4</td>
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* Inoculum for 10^4 protoplasts contained RNA1, 2, 3 preparation (0.5 μg) alone or in combination with transcripts of RNA4 (3 μg), or preparations of total soluble proteins from induced or uninduced E. coli (5 μg), purified rCP (5 μg) or native CP (5 μg). 5 μg of induced total soluble protein contains 1.4–1.7 μg rCP.
† Infection was assayed by an immunofluorescence assay using antibodies to AMV CP. Only infected protoplasts were detected.
‡ Data for Experiment 1 are the average of two independent replicates.
NT, Not tested.
resulted in infectivity. The activity of the rCP preparations increased with purification and/or concentration of the rCP to nearly the same activity as native CP. Binding of CP to AMV RNA is required for activity (Van der Vossen et al., 1994; Reusken et al., 1994; Yusibov & Loesch-Fries, 1995); therefore, the data in Table 1 suggest that rCP binds to AMV RNA even though the protein has an N-terminal extension.

In vitro assembly of empty particles and particles containing RNA

To determine if rCP would assemble into empty icosahedral particles as previously reported for native CP (Fukuyama et al., 1981), rCP (90 mg/ml) was extensively dialysed against pyrophosphate buffer. Spherical particles formed within 6 days of dialysis (Fig. 3A). Particles, negatively stained with uranyl acetate, had electron dense centres indicating that the uranyl acetate had penetrated the particles, suggesting that the particles were empty. The rCP in the assembled particles shown in Fig. 3A contained the N-terminal 37 amino acid extension. However, the particles were quite uniform in size and shape. Their average diameter (19 nm) was identical to the diameter of the trypsin-treated icosahedral particles assembled with native AMV CP by Fukuyama et al. (1981). This suggests that the N-terminal extension in rCP does not dramatically affect particle assembly.

To assemble particles containing AMV RNA4, rCP was mixed with RNA4 transcripts. Fig. 3B shows that a heterogeneous population of particles resulted. The majority of the negatively stained particles had electron transparent centres indicating that there was little empty space for penetration of the uranyl acetate, which suggests that the particles contained RNA. Two size classes of spherical particles were present, as well as, a few short bacilliform particles (Fig. 3B): 73% of the spherical particles had an average diameter of 19 nm and 27% had an average diameter of 26 nm. Dialysis of the particles against citrate buffer resulted in the formation of hexagonal crystals no larger than 0.3 mm. A few crystals were disrupted for examination of particles by electron microscopy; others were rinsed with 0.2 M-citrate buffer, pH 4.6 and disrupted for analysis of the encapsidated RNA by agarose gel electrophoresis. Fig. 3B shows that the crystals formed from the smaller uniform particles (left inset), which contained RNA that co-migrated with RNA4 transcripts upon electrophoresis (right inset). The in vitro assembly was repeated using
Fig. 4. Electron micrographs of particles assembled from rCP and RNA1 transcripts negatively stained with 2% uranyl acetate. Particles and aggregates formed during dialysis of a mixture of RNA1 transcripts and rCP (A, B) or native CP (C) against 75 mM-sodium pyrophosphate, pH 6-5. The electron micrographs are from separate experiments. Spindle-shaped aggregates are indicated by arrows. The bar indicates 100 nm for all three micrographs.

native AMV CP, isolated from virions, and RNA4 transcripts. The resulting particles were identical in size and heterogeneity to those shown in Fig. 3B (data not shown). The estimated radius of the interior of the empty T = 1 particle is 58 Å (Fukuyama et al., 1981), which suggests that a single hydrated transcript of RNA4 (898 nt) would fill approximately 77% of the interior. Thus, the small spherical particles shown in Fig. 3B are likely to be T = 1 particles, each containing a single molecule of RNA4.

To assemble particles containing AMV RNA1, the rCP was mixed with RNA1 transcripts and dialysed against pyrophosphate buffers as described above. No bacilliform particles were observed (data not shown). Therefore, the rCP was mixed with RNA1 transcripts at a ratio of 156 rCP:1 RNA (w/w) and dialysed against 50, 75 or 100 mM solutions of sodium pyrophosphate or NaCl, at pH 6 or 6-5. Dialysis against NaCl resulted in the formation of spherical particles that resembled T = 1 particles. Similar results were obtained with 50 or 100 mM-pyrophosphate, pH 6 or 6-5 (data not shown). However, the assembly products formed during dialysis against 75 mM-pyrophosphate were strikingly different. Small spheres, bacilliform particles and large aggregates were present. At pH 6-0, only a few short bacilliform particles were present (data not shown). However, at pH 6-5, bacilliform particles formed which were similar in size to native particles containing RNA1 (Fig. 4A, B). Spindle-shaped aggregates, some of which appeared to be made up of bacilliform particles and spherical particles, were also present (Fig. 4B, arrows). To determine if bacilliform particles could be made with native CP, RNA1 transcripts and CP isolated from virus particles were assembled under the same conditions that were used for rCP. As shown in Fig. 4C, only occasional short bacilliform particles were found. In a number of experiments, rCP consistently assembled virus-like bacilliform particles while native CP did not.

Crystallization and X-ray analysis of empty particles

The empty particles (Fig. 3A) were treated with trypsin to remove the N terminus of the rCP for crystallization as described by Fukuyama et al. (1981). Analysis of the trypsin-treated particles by SDS-PAGE indicated that all of the rCP was converted to a protein (21 kDa) that migrated faster than rCP (data not shown). Crystals were obtained using the conditions described by Fukuyama et al. (1981) as outlined in Methods. The crystals grew to a size of about 1.5 mm in 1 week and had a hexagonal shape as described by Fukuyama et al. (1981). The crystals diffracted X-rays to 5.5 Å resolution with occasional spots appearing as far as 5.0 Å. Film processing showed that the crystals belong to the hexagonal system (space group P63) with unit cell parameters a = 198.95; c = 311.25 Å.

Discussion

The goal of this study was to determine whether AMV CP expressed in E. coli (rCP) was similar to native CP in biological activity and in the assembly of virus particles in vitro. Our results indicate that the recombinant protein containing a 37 amino acid extension is active in infection and assembly. This suggests that the extension at the N terminus of the recombinant CP does not affect the accessibility of the N terminus for binding with the viral RNA. It also suggests that no post-translational modi-
fication, such as acylation of serine at position 2, which occurs in native AMV CP, is needed for biological activity.

When incubated under assembly conditions, AMV rCP formed empty spherical particles that were similar in diameter to those assembled from native CP (Fukuyama et al., 1981). Preliminary analysis of the X-ray diffraction data indicated that the empty particles are similar in size and surface properties to those previously investigated by Fukuyama et al. (1983).

AMV CP will encapsidate nucleic acids resulting in variously shaped particles (Hull, 1970; Lebeurier et al., 1971; Driedonks et al., 1978). The availability of AMV RNA transcripts made it possible to provide pure preparations of the smallest and largest AMV RNA for the assembly reaction. Particles containing AMV RNA4 formed under conditions similar to those for empty rCP particles. Most of the particles appeared to contain RNA4 as determined by electron microscopy. We expected to observe some empty particles because rCP was present at great molar excess in relation to the preparations of the smallest and largest AMV RNA for particles to form. The electron micrographs of particles AMV RNA4 3' end and the rCP permitted few empty expected to observe some empty particles because rCP when rCP was assembled with RNA1 transcripts, was much more competent in the assembly of bacilliform particles than was CP isolated from bacteria characterizing interaction of AMV CP and AMV RNA 3' ends in the context of assembly.

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