Expression and biochemical analyses of the recombinant potato virus X 25K movement protein

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Abstract The 25K movement protein (MP) of potato virus X (PVX) is encoded by the 5′-proximal gene of three overlapping MP genes forming a 'triple gene block'. The PVX 25K MP (putative NTPase-helicase) has been synthesized in *Escherichia coli* as a recombinant containing a six-histidine tag at the amino terminus. The His-tagged 25K protein was purified in a one-column Ni-chelate affinity chromatography procedure. In the absence of any other viral factors, this protein had obvious Mg^{2+}-dependent ATPase activity, which was stimulated slightly (1.7–1.9-fold) by various polynucleotides. Like other viral proteins possessing ATPase-helicase motifs and many plant viral movement proteins, the PVX 25K MP was able to bind nucleic acids in vitro. The RNA binding activity of the 25K MP was pronounced only at very low salt concentrations and was independent of its ATPase activity.

Key words: Plant RNA virus; Movement protein; ATPase; RNA binding

1. Introduction

Potexviruses form a large group of positive-strand RNA-containing plant viruses belonging to the 'Sindbis-like' supergroup. The nucleotide sequences of many potexvirus genomes including those of potato virus X (PVX) (type member of the group), white clover mosaic virus, foxtail mosaic virus (FMV) and *Plantago asiatica* mosaic virus have been determined [1–4]. The PVX genome contains five open reading frames (ORFs): the 5′ proximal ORF coding for the RNA-dependent RNA polymerase (165K protein); three internal partially overlapping ORFs encoding the 25K, 12K and 8K proteins and the 3′ proximal ORF corresponding to the coat protein gene [5]. According to the gene arrangement and protein sequences encoded, three internal ORFs in the PVX genome closely resemble similar triple gene blocks (TGB) in the genomes of carlaviruses, hordeiviruses and some furo-like viruses [6–8]. Three proteins encoded by TGB (TGBp1, TGBp2 and TGBp3) were found to be involved in the virus movement over the plant tissues, i.e. they are the movement proteins (MPs) [9–12]. Despite significant diversity in size, the TGBp1 proteins encoded by distantly related viruses exhibit obvious sequence similarity and contain sequence motifs of some ATPases and helicases [5,13,14]. ATPase activity of the PVX 25K MP counterparts in FMV and barley stripe mosaic hordeivirus (BSMV) has been recently found ([15], A.O. Jackson, personal communication). It has been shown also that these TGB proteins and many other plant viruses MPs are able to bind RNA in vitro [15–22].

In this paper, we show that the purified 25K MP (p25) exhibits in vitro the ATPase activity that is stimulated slightly by polynucleotides. The RNA binding activity of the 25K protein is independent of the presence of ATP and is drastically lower than the RNA binding activity of some other viral MPs.

2. Materials and methods

2.1. Constructing a vector for superexpression

All recombinant DNA procedures were carried out by standard methods [23]. *E. coli* strains DH5α and M15[pREP4] were used for the cloning of created constructs and for superexpression, respectively. In order to fuse the 25K protein gene to the expression vector pQE-9 (QIAGEN), the PVX cDNA fragment (nucleotides 4486–5251) was amplified by PCR using as template the plasmid pXT7-25 [24] and as primers the oligodeoxynucleotides 25P-Nco-ccggatccATGGATATTCTCATCATTAGTT corresponding to the start of the 25K protein gene with upstream *Bam*HI site (in lower case) and 12M-AGAAAGCAGAAAGGTAATTGAAACT complementary to the internal part of the 12K protein gene (Fig. 1). After digestion with *Bam*HI, the fragment was ligated with a *Bam*HI-*Hin*dIII (filled) cleaved pQE-9 (QIAGEN), yielding pQ25.

2.2. 25K protein expression in bacteria and its purification

Escherichia coli strain M15 transformed with the recombinant vector pQ25 was grown at 37°C in liquid culture until an OD_{600} of 0.8–0.9 was reached. Expression of the p25 was induced with 1 mM IPTG followed by growth for 3 h at 37°C. The purification of p25 from cultures followed a general procedure described by the manufacturer (QIAGEN) for denaturing Ni-NTA chromatography.

2.3. ATP hydrolysis assays

ATPase activity assays were performed with the purified p25 (0.25–1.0 μg) in buffer containing 20 mM HEPES, pH 7.5; 10% glycerol; 1 mM EDTA; 5 mM $MgCl_2$; 0.1 mM ATP and 0.5 μCi [γ-^{32}P]ATP in a final volume of 10 μl. When indicated, the nucleic acids were included in the reaction mixture at different concentrations. Mixtures were incubated for 2 h at 37°C and the reaction was stopped by adding EDTA to a final concentration of 20 mM. 1 μl of each probe was spotted onto a PEI-cellulose F-coated plastic sheet (Merck) and developed by thin-layer chromatography using 0.15 M formic acid and 0.15 M LiCl. Products of hydrolysis were visualized by autoradiography of the dried sheet. Samples of [γ-^{32}P]ATP incubated in the same buffer with or without apyrase (Sigma) served as the ATP and P_i mobility markers, respectively.

To estimate the enzymatic activity, two methods were used. (1) Regions of the sheet corresponding to ATP and P_i were excised and counted in a toluene scintillation cocktail to determine the percentage of ATP hidrosis. ATP decay background was subtracted from each figure. (2) After incubation, unreacted ATP was precipitated by addition of 300 μl of 7.5% activated charcoal in 50 mM HCl–5 mM H_3PO_4; the mixtures were vortexed and allowed to stand for 5 min, then charcoal was centrifuged in a microcentrifuge for 10 min and

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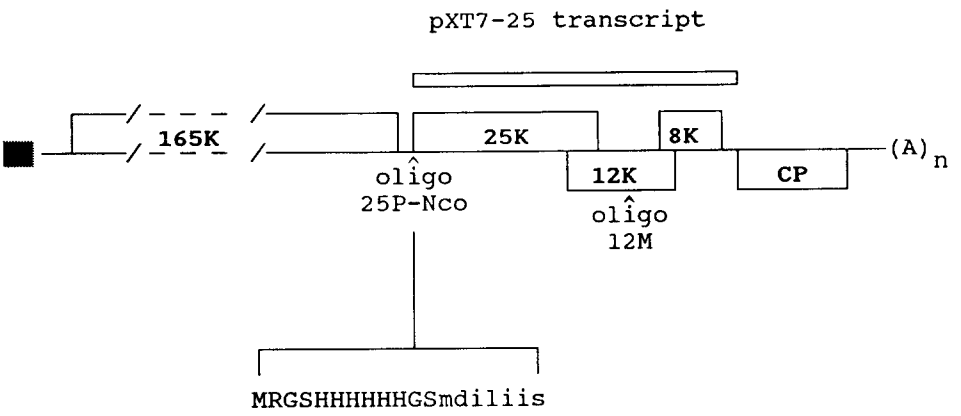

Fig. 1. Organization of the PVX genome, structure of the virus-specific insert in pQ25 and the deduced N-terminal amino acid sequences of His-tagged 25K MP produced from pQ25. The positions of the primers for PCR and the transcript for RNA binding assay are shown.

half of the supernatant was analyzed by Cerenkov counting for free $^{32}P_i$ liberated by hydrolysis of the labeled ATP.

2.4. RNA binding assays

RNA binding experiments were performed with filter-bound proteins according to previously published protocols [25]. The purified recombinant p25 and the TMV-coded 30K MP (p30) taken as a control were separated by electrophoresis in a 15% SDS-PAGE and electroblotted onto nitrocellulose membranes. Membranes were washed twice for 30 min each with an aqueous solution of 6 M urea with 0.1% Tween 20. Membrane-bound proteins were renatured in buffer R containing 20 mM Tris-HCl, pH 7.5; 0.2 g/l BSA, 0.2 g/l Ficoll, 0.2 g/l polyvinylpyrrolidone, NaCl and Mg^{2+} at different ratios (as noted in the legend to Fig. 4) for 1 h with 2–3 changes of buffer. After renaturation the membranes were incubated at room temperature with γ-^{32}P-labeled RNA transcript (1×10^6–1.5×10^6 dpm/ml) in 2.0 ml of buffer R of the same salt composition during 1 h. Membranes were washed 3–4 times for 30 min each with buffer R, dried and autoradiographed. Labeled single-stranded RNA was synthesized by T7 RNA polymerase in the presence of [α-^{32}P]UTP from linearized pXT7-25 template (Fig. 1) [24].

To examine the RNA binding properties of p25, the protein-dependent retention of ^{32}P-labeled pXT7-25 RNA transcripts on nitrocellulose filters was used. The reaction mixture, containing, in a total volume of 50 ml, 20 mM HEPES (pH 7.5), 5 mM $MgCl_2$ and different concentrations of NaCl as indicated, approximately 5–10 ng of labeled RNA and 500 ng p25, p30 or BSA taken as a control, was incubated at 0°C for 1 h. After incubation, the samples were diluted with 1 ml of cold washing buffer: 20 mM Tris-HCl, pH 7.5; 2.5 mM $MgCl_2$; 0.1 mM EDTA, and applied to nitrocellulose filters under vacuum. The test tubes were washed once, and then the filters were washed twice more with 1 ml cold washing buffer. The filters were dried, and the radioactivity was measured by liquid scintillation counting.

3. Results and discussion

3.1. Expression of the 6×His-tagged 25K protein in bacteria and its purification

In order to obtain the PVX 25K MP in preparative amounts, we cloned its coding sequence in the expression

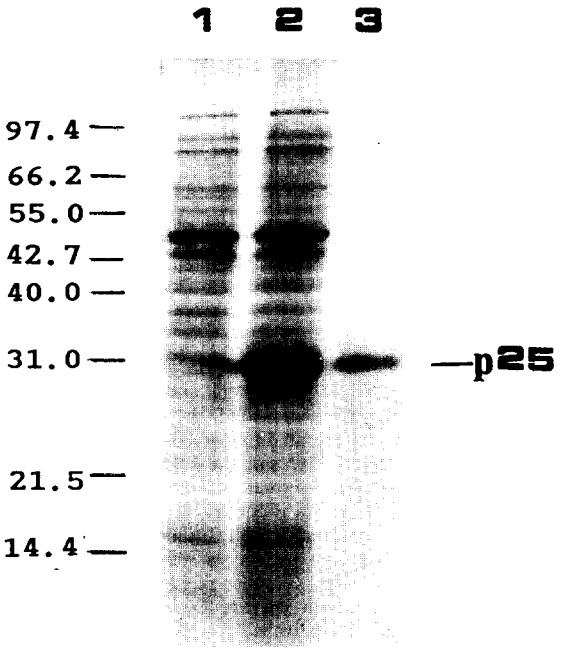

Fig. 2. Analysis of the expression in *E. coli* and purification of the PVX 25K MP by SDS-PAGE. Lanes: 1, total protein extract without induction with IPTG; 2, total protein extract after induction with IPTG; 3, the preparation of the recombinant p25 after the purification (Coomassie blue staining). The sizes of molecular weight markers are shown in the left margin (kDa). The arrow in the right margin points at p25.

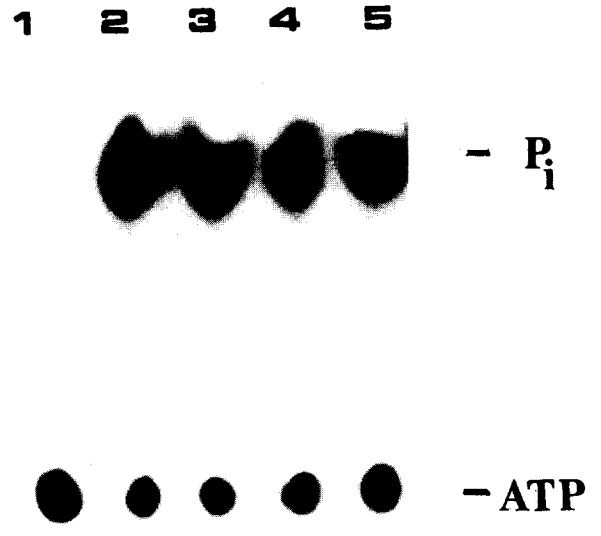

Fig. 3. Mg^{2+}-dependent ATPase activity of the recombinant 25K protein PVX. Autoradiograph of ATPase products generated by purified p25 in the absence (line 1) or in the presence (lanes 2–5) of different concentrations of Mg^{2+} (2.5; 5.0; 7.5 and 10.0 mM Mg^{2+} respectively). The mobilities of ATP and P_i are shown in the right margin.

plasmid pQE-9 (QIAGEN). The design of the resulting plasmid pQ25 was such that signals for bacterial expression directed the synthesis of the 25K protein with the insertion of 12 additional amino acids (including 6 His residues) at the N-terminus (Fig. 1). The total cellular proteins from the recombinant bacteria uninduced or induced with IPTG were analyzed by SDS-PAGE for expression of the 25K MP (Fig. 2, lanes 1, 2). The apparent molecular weight of the major protein expressed by *E. coli* cells was in good agreement with the values found for the labeled 25K protein produced in protein-synthesizing cell-free systems [24]. Lysis of the M15 cells released a large proportion of the 25K MP into the soluble fraction. The 25K protein was purified from bacterial lysates by a one-step procedure using Ni-NTA agarose columns. Under these conditions, more than 80% of protein was eluted from the column. Fractions containing the 25K MP and having negligible amounts of the contaminating bacterial proteins were pooled (Fig. 2, lane 3). Protein yield ranged near 3 mg per 200 ml culture of *E. coli* M15.

3.2. ATPase activity of the 25K MP

To examine whether the purified PVX 25K MP contains an associated ATPase activity, the products of ATPase hydrolysis of [γ-^{32}P]ATP were separated by thin-layer chromatography. The p25 preparations manifested the ATPase activity which was dependent on the presence of Mg^{2+} (Fig. 3). To rule out the possibility that the observed ATPase activity was due to minor amounts of contaminating bacterial proteins that might be present in the p25 preparations, we tested the preparations of two other histidinylated recombinant proteins purified by the same method. The 30K MP of TMV and dihydrofolate reductase did not display the magnesium-dependent ATPase activity. Likewise, no ATPase activity was observed in the preparations eluted from the column loaded with the lysate of M15 cells carrying pQE30 without the 25K gene insert (data not shown).

The ATPase activity of the 25K MP was slightly (1.5–1.9-fold) stimulated by poly(A)$_{60-80}$, PVX RNA and TMV RNA (Table 1). A similar level of nucleic acid stimulation of NTPase activity was found in the plum pox virus RNA-helicase C1 protein [26,27].

3.3. Nucleic acid binding activity of the 25K MP

The single gene-coded MPs of plant viruses differ significantly in efficiency of RNA binding [18–22]. It was suggested that TGBp1 may contain an analog of the RNA binding domain of such MPs [28,29]. Indeed, it was demonstrated that the FMV 26K TGBp1, beet necrotic yellow vein virus (BNYVV) 42K TGBp1 and BSMV 58K TGBp1 were capable of forming stable complexes with RNA molecules in vitro [15–17]. Moreover, BSMV TGBp1 forms RNP complexes in plants at early stages of viral infection [30].

Table 1
Effects of RNAs on the ATPase activity of the PVX 25K MP

Polynucleotide (μg/ml)	Relative ATPase activity[a]
None	1.0
Poly(A)$_{60-80}$	
60	1.5
250	1.7
TMV RNA	
50	1.7
100	1.9
PVX RNA	
30	1.6
100	1.8

[a]Hydrolysis of [γ-^{32}P]ATP by p25 was measured as the release of P_i in the absence or presence of the indicated quantities of polynucleotides. The activity without added RNA was defined as 1.0, all other activities were normalized to this value.

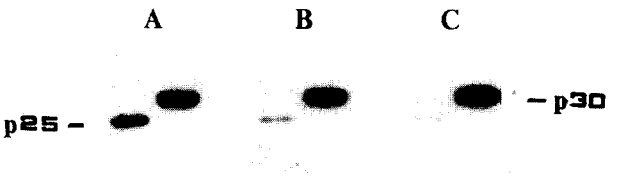

Fig. 4. Salt dependence of RNA binding activity of the 25K protein in Northwestern binding assay. 2 μg of PVX p25 and TMV p30 was subjected to 15% SDS-PAGE, transferred to nitrocellulose strips and probed with ^{32}P-labelled RNA transcript in different salt conditions. A, incubation without $MgCl_2$ and NaCl; B, incubation with 5 mM $MgCl_2$ without NaCl; C, incubation with 5 mM $MgCl_2$ and 50 mM NaCl. The positions of the PVX 25K MP and the TMV 30K MP are indicated.

To examine the RNA binding capacity of p25, we used two experimental approaches: Northwestern binding assay and protein-dependent retention of RNA on nitrocellulose filters. In the first series of experiments the PVX 25K MP and the TMV 30K MP (taken as a control) were electrophoresed in SDS-PAGE, transferred to nitrocellulose filters and probed with ^{32}P-labeled RNA transcript. In vitro RNA binding activity of the PVX 25K MP could be detected readily only in the absence of NaCl (Fig. 4A). The efficiency of the 25K MP RNA binding decreased progressively after addition of 5 mM $MgCl_2$ (Fig. 4B) and 50 mM NaCl (Fig. 4C). The RNA binding activity of the PVX p25 was dramatically lower than that of the TMV 30K MP (Fig. 4) taken as the most thoroughly characterized viral MP [18]. Moreover, the salt stability of the 25K MP-RNA complexes was significantly lower than that of the complexes formed between RNA and TGBp1 MPs of other TGB-containing viruses including FMV [15], BNYVV [17] and BSMV (our unpublished data). The efficiency of the PVX 25K MP in RNA binding was independent of the presence or absence of ATP in the incubation mixture (data not shown). Therefore, the ATPase activity of the 25K MP is not required for 25K MP-RNA complex formation.

In a separate series of experiments the RNA binding activity of the 25K MP was examined by the nitrocellulose binding assay. The data obtained in these experiments were principally the same as in the Northwestern binding (data not shown).

The reasons for the difference in RNA binding activities between the PVX 25K MP and the MPs encoded by other viruses remain obscure. One can speculate that the PVX 25K MP ATPase activity involved in cell-to-cell transport function may require only a weak and transient interaction with RNA molecules whereas the RNA binding of other viral MPs is strong and cooperative. It can be proposed also that such interaction of the PVX 25K MP with RNA is facilitated and/or stabilized by other viral and/or cellular proteins.

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