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Natively unfolded nucleic acid binding P8 domain of SeMV polyprotein 2a affects the novel ATPase activity of the preceding P10 domain

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ABSTRACT

Open reading frame (ORF) 2a of *Sesbania mosaic virus* (SeMV) codes for polyprotein 2a (Membrane anchor-protease-VPg-P10–P8). The C-terminal domain of SeMV polyprotein 2a was cloned, expressed and purified in order to functionally characterize it. The protein of size 8 kDa (P8) domain, like viral protein genome linked (VPg), was found to be natively unfolded and could bind to nucleic acids. Interestingly, P10–P8 but not P8 showed a novel Mg^{2+} dependent ATPase activity that was inhibited in the presence of poly A. In the absence of P8, the ATPase activity of the protein of size 10 kDa (P10) domain was reduced suggesting that the natively unfolded P8 domain influenced the P10 ATPase.

1. Introduction

Sesbania mosaic virus (SeMV) is a sobemovirus that contains 4149 nucleotides long RNA genome with a viral protein genome linked (VPg) attached to its 5′ end [1]. Of the four open reading frames (ORFs), the two central ORFs 2a and 2b are translated into polyproteins. ORF 2a codes for polyprotein 2a with a domain arrangement of membrane anchor-serine protease-VPg–P10–P8 (Fig. 1a) [2]. ORF 2b is translated by −1 ribosomal frameshifting into polyprotein 2ab (MA-protease-VPg-RNA dependent RNA polymerase). The N-terminal serine protease domain was shown to be responsible for the processing of the polyproteins into functional domains [2,3]. Our recent study demonstrated that the removal of membrane anchor via the cleavage at E132-S133, is essential for the cleavage at E498–S499 site between protein of size 10 kDa (P10) and protein of size 8 kDa (P8) domains [2].

The genome organization and strategies of expression of ORF 2a and 2b of sobemoviruses are quite similar to those of *Luteoviridae* and also to fungus infecting barnaviruses [4–7]. Interestingly, the mature form of VPg gets processed from the polyprotein (Protease-VPg-C-terminal domain) in all these viruses. Preservation of an additional domain downstream of VPg across viral families indicates its essential role in viral life cycle.

With an aim to identify the functional role of P10 and P8, the domains beyond VPg in SeMV, the present study was undertaken. *Escherichia coli* expressed and purified P8 was found to be natively unfolded and bound to RNA and DNA in vitro. Interestingly, P10 and P10–P8 (P18) but not P8 showed a Mg^{2+} dependent novel ATPase activity. P18 was enzymatically more active than P10 suggesting that natively unfolded P8 could activate P10 ATPase.

2. Materials and methods

2.1. Oligonucleotide primers

The oligonucleotide primers used for gene amplification were custom made from Sigma Chemicals.

2.2. Cloning, expression and purification

The region corresponding to P10, P18 was amplified using 2a pRSET C clone as template and specific primers.

P10 sense primer: CTA**GCTAGC**CATATGACCGTCGCTGTTGAG

P10 anti sense primer: CGGGATCCTCATTCCTGCTTGTAATAACA AGG

P8 anti sense primer: CGGGATCCTCAGTAACACAGAGAGCAA CAAG

Abbreviations: SeMV, *Sesbania mosaic virus*; ssRNA, single-stranded RNA; ORF, open reading frame; VPg, viral protein genome linked; MA, membrane anchor; P10, protein of size 10 kDa; P8, protein of size 8 kDa

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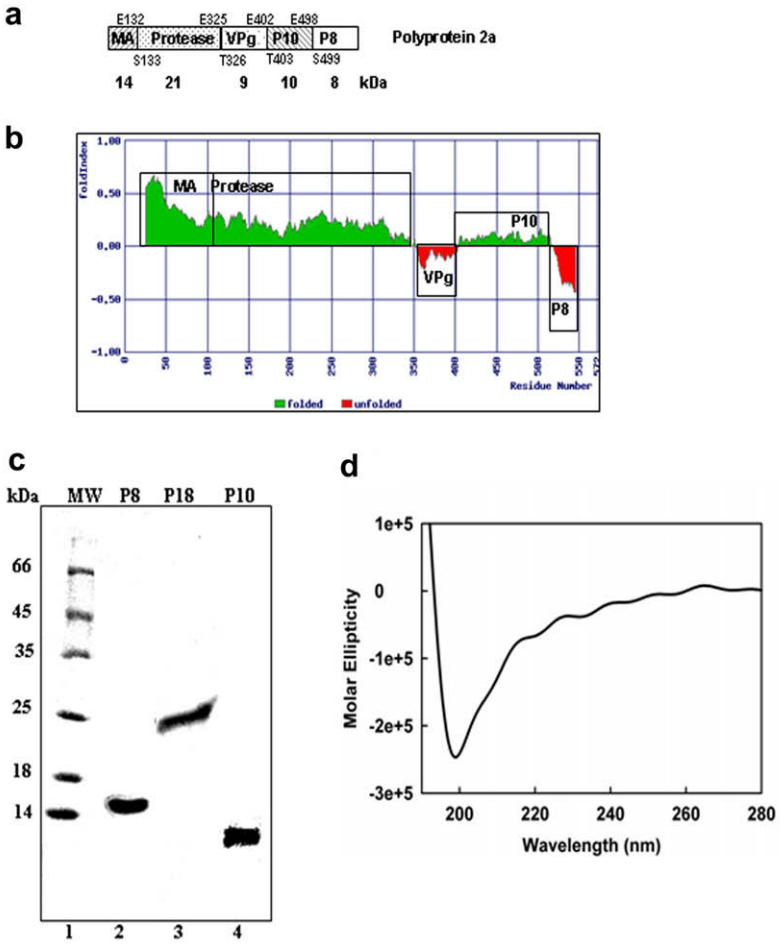

Fig. 1. (a) Schematic representation of the domain arrangement of SeMV polyprotein 2a. Sites of cleavage and the expected size of each domain have been indicated. (b) Outputs obtained using the FoldIndex program for polyprotein 2a sequence. Prediction of unfolded protein was performed using the program FoldIndex with the default values. The boundaries of each domain have been boxed. (c) 15% SDS–PAGE showing P8, P18 and P10 proteins purified by Ni-NTA affinity chromatography. (d) The Far-UV CD spectrum of P8 protein. The ellipticity was monitored from 190 to 280 nm using 400 μg/ml of the protein in 20 mM Tris–HCl pH 8.0.

(*Nhe*I site is denoted in bold and *Bam*HI site is underlined). PCR was carried out using High Fidelity Phusion polymerase (Finnzymes) and the product cloned at the *Nhe*I and *Bam*HI sites in pRSET C vector. The clone was confirmed by DNA sequencing.

P8 was purified as described previously [2]. Briefly, P8 clone was transformed in *E. coli* BL21 (DE3) pLys S cells and the protein was expressed upon induction with 0.3 mM IPTG. The cell pellet was resuspended in the sonication buffer (50 mM Tris–HCl pH 8.0, 500 mM NaCl, 0.1% Triton X-100, 5% glycerol, 10 mM L-arginine) and sonicated. The protein was purified from the soluble fraction by Ni-NTA affinity chromatography (Novagen) according to the manufacturer's instructions. The recombinant P18 or P10 clones were transformed in *E. coli* BL21 (DE3) cells and the proteins were expressed upon induction with 0.3 mM IPTG purified by Ni-NTA chromatography as described for P8.

2.3. Circular dichroism

Far-UV CD spectrum was obtained for purified P8 protein as described in [8].

2.4. Bioinformatic analysis

The software FoldIndex©, (http://bip.weizmann.ac.il/fldbin/findex#info) available at the Weizmann Institute of Science was used for unfolded protein prediction. The FoldIndex© analysis of 2a amino acid sequence was performed using default values. The analysis was also carried to identify the presence of any RNA binding sites in polyprotein 2a, using the RNABindR software available at (www.bindr.gdcb.jastate.edu/RNABindR).

2.5. Nucleic acid binding studies

Purified P8 was incubated with genomic RNA (1 μg) or dsDNA (VPg–P10–P8 PCR product, 125 ng) in the presence of 1X binding buffer (50 mM NaCl, 25 mM Tris–HCl pH 7.4, 25 mM KCl, 2 mM EDTA) for 30 min at room temperature (RT). The sample was run on 0.8% agarose gel (in 1X Tris Borate buffer, pH 8.0). The gel was stained with ethidium bromide and exposed to UV light.

2.6. ATPase assay

A standard ATPase assay was performed by incubating 0.6 μg of protein in a buffer containing 50 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 2 mM DTT, 0.5 mg/ml BSA, 0.1 μCi γ-P^{32} ATP, 100 μM cold ATP at 37 °C for 30 min. The reaction was stopped by quick chilling in liquid N_2. ^{32}Pi release was estimated by spotting 1 μl reaction mixture on polyethyleneimine (PEI) cellulose F plates (Merck) followed by thin layer chromatography (TLC) using 0.15 M formic acid, 0.15 M LiCl and 1 mM EDTA pH 8.0 as solvent. The plates were exposed to phosphorimager and the intensity of ^{32}Pi spots was quantified. For kinetic studies, 0.1 μCi γ-P^{32} ATP and 2–500 μM cold ATP

was incubated with 0.35 μg of protein in the assay buffer. The experiments were performed in triplicates and the average intensity values were used to determine activity (v), calculated using the formula given below:

$$\text{Activity, pmoles/min/}\mu\text{g } (v) = [\text{Fc} * \text{picomoles of cold ATP}]/[\text{time of incubation} * \mu\text{g of protein}]$$

where Fc, Fractional Cleavage = [Intensity of ^{32}Pi]/[Intensity of ^{32}Pi + Intensity of γ-P^{32}ATP].

2.7. Immunodepletion studies

P18 protein (0.6 μg) was incubated with 1 μg of the anti P8 polyclonal antibodies at 4 °C for 1 h followed by binding to the protein A sepharose beads at 4 °C for 1 h. The beads were centrifuged at 1000 rpm and the supernatant was used for ATPase assay. The anti P8 antibodies interact with P18 via the P8 domain as these antibodies do not cross react with purified P10 alone.

3. Results and discussion

3.1. Bioinformatic analysis

SeMV polyprotein 2a has a domain arrangement as shown in Fig. 1a [2]. In our earlier studies, VPg was shown to be natively unfolded [9]. Therefore, bioinformatic analysis was carried out for full length polyprotein 2a using the FoldIndex© program. Interestingly, the polyprotein 2a showed an alternating arrangement of folded and unfolded domains, with protease and P10 regions being folded and VPg and P8 unfolded (Fig. 1b).

3.2. CD spectrum analysis

Random coil structures exhibit a minimum at 200 nm on a Far-UV CD spectrum [10]. P8 protein was purified as described in Section 2 (Fig. 1c, lane 2). The purified P8 protein showed mass abnormality on the SDS–PAGE, owing to its highly basic nature (predicted pI value of 11.75) [2]. The higher mass could not be because of dimerization as purified P18 showed abnormal mass of 25 kDa and not 36 kDa (Fig. 1c, lane 3). The Far-UV CD spectrum of P8 showed a negative ellipticity at 200 nm (Fig. 1d), confirming that like VPg, P8 too was natively unfolded.

3.3. Nucleic acid binding property

Highly positively charged unfolded P8 domain could possibly bind to nucleic acids. Bioinformatic analysis was performed for both polyprotein 2a and P8 domain, using the RNABindR software available at (www.bindr.gdcb.jastate.edu/RNABindR), in order to identify the presence of any RNA binding sites. The results revealed that in the entire polyprotein 2a, only P8 (residues 34–68) had a RNA binding region (data not shown). In order to test this, the purified P8 was incubated with genomic RNA in the binding buffer and analyzed by agarose gel electrophoresis. With increasing amounts of P8 (0.5–5 μg), the nucleoprotein complex showed increase in the retardation and at 5 μg of P8, the complex remained in the well (Fig. 2a). It was of interest to see if P8 could also bind to DNA. Increasing amounts of the protein (0.5–5 μg) was incubated with 125 ng of VPg–P10–P8 PCR product (743 bp). As observed in Fig. 2b, there was an increase in the retardation of the DNA-P8 complex with increasing amounts of P8, indicating that P8 could also bind to the dsDNA. Further, P8 could bind to other non-specific RNA and DNA such as poly A and M13 primer (data not shown), suggesting that binding of P8 to nucleic acids could be non-specific.

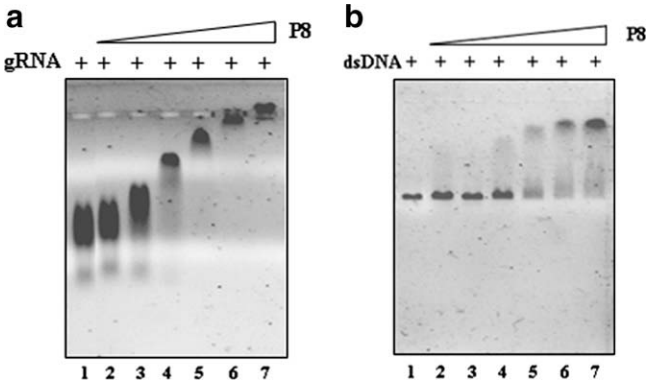

Fig. 2. Gel retardation assay for nucleic acid binding by purified P8 protein. (a) Purified P8 (0.5, 1, 2, 3, 4 and 5 μg, lanes 2–7) was incubated with genomic RNA (1 μg) in the presence of 1X binding buffer (50 mM NaCl, 25 mM Tris–HCl pH 7.5, 25 mM KCl, 2 mM EDTA) for 30 min at RT. The nucleoprotein complex was visualized after electrophoresis on a 0.8% agarose gel, followed by staining with ethidium bromide. Lane 1-RNA alone. (b) 125 ng of the DNA (VPg–P10–P8 PCR product) was incubated with 0.5, 1, 2, 3, 4 and 5 μg of P8 (lane – 2, 3, 4, 5, 6, 7, respectively), and the samples were analyzed by agarose gel electrophoresis as described above. Lane 1-DNA alone.

These results suggest that P8 can bind to single or double stranded nucleic acid.

Such a nucleic acid binding region has also been identified at the C-terminus of VPg in polyprotein P1 of *Potato leafroll virus* (*Luteovirus*) and the polyprotein 2a of *Cocksfoot mottle virus* [11,12]. Together, these findings suggest that region at the C-terminus of VPg in the polyprotein may play an essential role in the viral life cycle. For example, it is possible that polyprotein 2a could bind to the gRNA via C-terminal P8 RNA binding domain that might aid in correctly positioning the natively unfolded VPg to get uridinylated by RdRp, following which the P10–P8 domain could get cleaved off. Further studies need to be carried out to establish any such possibility. In 40% of the RNA binding proteins, the RNA binding sites overlap with the nuclear localization signal (NLS). Prediction shows that P8 may have a bipartite NLS (data not shown). It would be interesting to see if P8 or its precursors could enter the nucleus of the host cell and act as transcriptional regulators.

3.4. ATPase activity

Nucleic acid binding property of P8 indicates the possible role of P8 or its precursors in viral replication or movement. Since energy requirement is an essential prerequisite in these processes, it was of interest to see if P8 could provide for the same by hydrolyzing ATP. The ATPase assay was performed with the purified P8 protein as described in Section 2. As seen in Fig. 3a, when increasing amounts of protein (0–0.8 μg) was incubated with γ-^{32}P ATP, no ^{32}Pi release was observed, suggesting that P8 was devoid of ATPase activity (Fig. 3a, lanes 1–4). On the other hand 0.5 μg calf intestinal alkaline phosphatase used as positive control showed release of ^{32}Pi (Fig. 3a, lane 5).

Since P10–P8 (P18) is a precursor of P8 in the polyprotein processing [2], presence of ATPase activity was also tested for this protein. The protein was expressed and purified as described in Section 2 (Fig. 1c, lane 3). Purified His tagged P18 also showed mass abnormality (25 kDa instead of 19 kDa). When increasing amounts of protein (0–0.8 μg) was incubated with γ-^{32}P ATP, a dose dependent increase in the release of ^{32}Pi was observed (Fig. 3b). Further, as seen in Fig. 3c, lane 2, P18 immunodepleted with anti P8 antibodies showed no release of ^{32}Pi as against the control P18 (Fig. 3c, lane 1), confirming that the ATPase activity was inherent to P18. Most of the viral ATPases are known to be stimulated by nucleic acids [13,14]. Therefore ATPase assay with

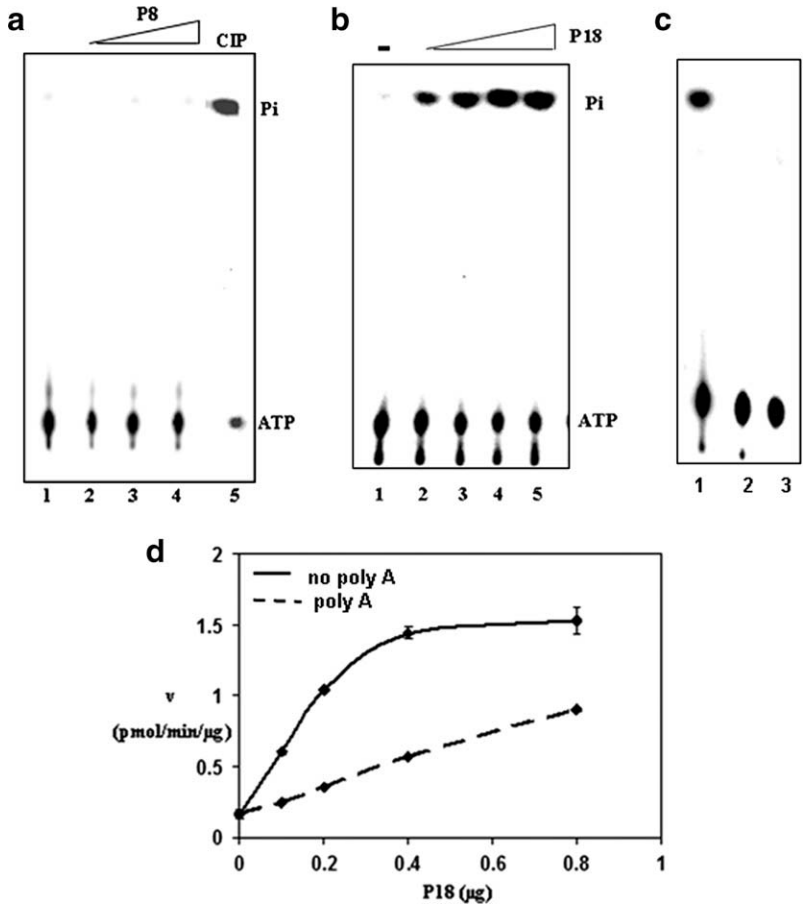

Fig. 3. (a) ATPase assay. The assay was performed by incubating 0, 0.2, 0.4, 0.8 μg (lanes 1–4) of P8 protein in a buffer containing 50 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 2 mM DTT, 0.5 mg/ml BSA, 0.1 μCi γ-P^{32} ATP, 100 μM cold ATP at 37 °C for 30 min and ^{32}Pi was separated on PEI-Cellulose plates by TLC. Lane-5 ATPase assay with calf intestinal alkaline phosphatase (CIP). (b) ATPase activity with 0.1, 0.2, 0.4, 0.8 μg of P18 protein (lanes 2–5). (c) Immunodepletion studies. Lane 1-ATPase assay with P18 (0.6 μg); 2-immunodepleted P18; 3 – no protein. (d) ATPase assay was performed with increasing amounts of P18 in the absence and presence of 0.5 mg/ml of poly A. Activity (v, pmol/min/μg) was plotted as a function of amount of P18.

P18 was carried out in the absence and presence of poly A (0.5 mg/ml). As seen in Fig. 3d, P18 ATPase was substantially inhibited in the presence of poly A. A similar kind of inhibition was also reported for the ATPase activity of Human coronavirus 229E nsp13 and poliovirus protein 2C, though the reason for the inhibition was unclear [15,16]. The inhibition of P18 ATPase by poly A could be due to the binding of poly A to P8 domain. Since, gRNA incubated with P10 did not show any retardation in EMSA (data not shown), it is unlikely that P10 could be involved in such an inhibition by poly A.

Time course experiments revealed that the activity was linear up to 20 min (Fig. 4a). The optimum activity was observed with 50 mM Tris–HCl buffer pH 7.5 and at 37 °C (data not shown). The activity was inhibited with increasing concentrations of NaCl (Fig. 4b), suggesting involvement of ionic interactions in the binding of the substrate. The activity was also inhibited in the presence of EDTA (Fig. 4c), suggesting the requirement of divalent metal ions. Studies on Mg^{2+} ion dependence showed maximum activity at 5 mM $MgCl_2$ (Fig. 4d) and other divalent metal ions (Mn^{2+}, Zn^{2+}, Ca^{2+}) inhibited the activity beyond 1–2 mM (Fig. 4d), suggesting that P18 is a Mg^{2+} dependent ATPase.

The assay was also performed in the presence of 1 mM excess of cold NTPs and dNTPs along with γ-p^{32} ATP. Fig. 4e shows that γ-p^{32}ATP was competed by all the NTPs and dNTPs, to a greater extent by ATP and dATP. This suggests that P18 may be able to utilize other substrates.

3.5. Functional domain

As P8 by itself showed no ATPase activity and P18 did, this raised the question if the functional ATPase domain resided in the P10 sequence. The region corresponding to P10 was cloned in pRSET C vector, expressed and purified as described in Section 2. Fig. 1c, lane 4 shows the purified P10 protein. As seen in Fig. 5a with increasing amounts of the P10 (0–0.8 μg), a dose dependent increase in the release of ^{32}Pi was seen suggesting that unlike P8, P10 alone could hydrolyze ATP. The ATPase activity of P18 and P10 was performed with increasing concentrations of ATP. The Lineweaver–Burk plot ($1/v$ vs $1/[ATP]$) was obtained (Fig. 5b) and the kinetic parameters were calculated. The Km for P18 and P10 was 90.9 μM and 27.7 μM and V_{max} was 4.29 and 1.71 pmol/min/μg, respectively. k_{cat} for P18 and P10 was 0.0815 and 0.0188 min^{-1}, respectively. In the presence of P8 domain, there was an increase in the k_{cat} of P10 ATPase by 4.3-fold, V_{max} by 2.5-fold and K_m by three-fold. In other words, the ATPase activity of P10 domain was influenced by the natively unfolded P8 domain. Interestingly, in SeMV, the serine protease domain is also rendered active by VPg, yet another natively unfolded domain present at its C-terminus [9]. More intriguing is the fact that, like VPg P8 is also present at the C-terminus of P10. However, VPg present at the N-terminus of RdRp has been shown to have inhibitory effect on the in vitro polymerase activity of the later (unpublished observations). The alternating arrangement of folded and unfolded domains in SeMV polyprotein

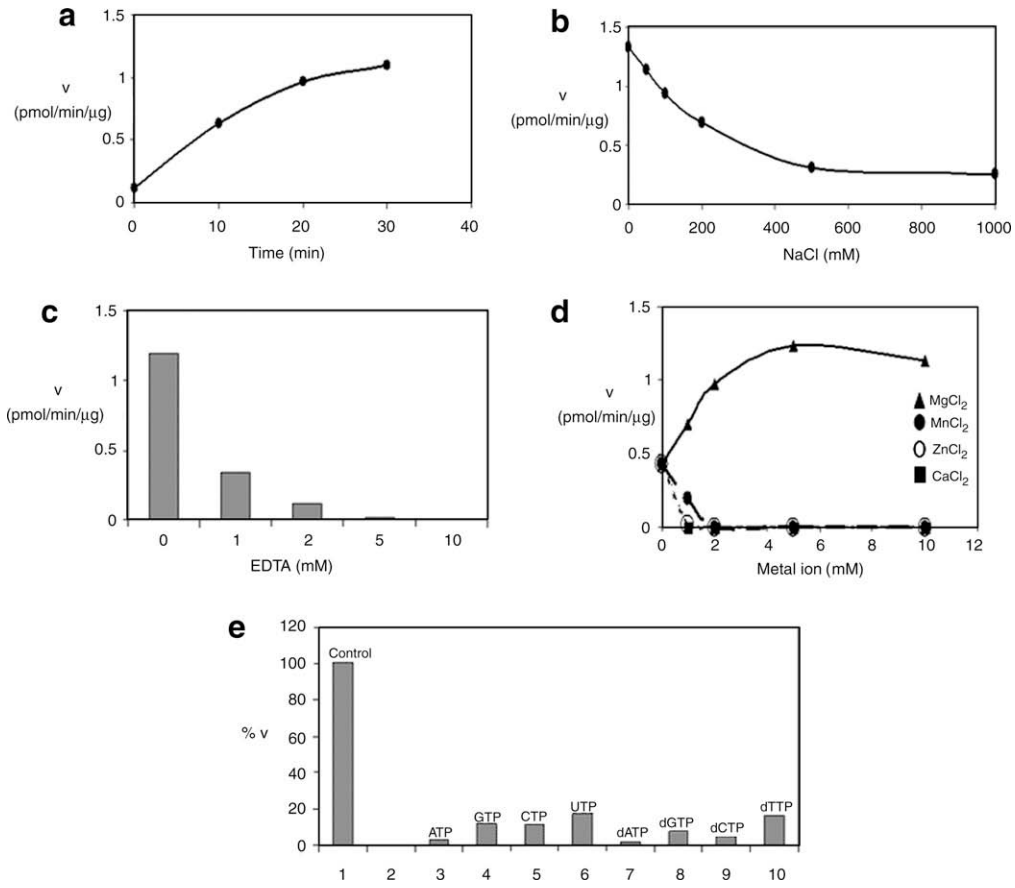

Fig. 4. ATPase assay was performed with 0.6 μg of P18 under different assay conditions. Activity (v, pmol/min/μg) was plotted against various parameters as indicated. (a) ATPase assay performed for different time intervals (0, 10, 20, 30 min). (b) ATPase assay performed at different NaCl concentrations (0, 50, 100, 200, 500, 1000 mM). (c) ATPase assay performed at different EDTA concentrations (0, 1, 2, 5, 10 mM). (d) ATPase assay was performed in presence of different divalent metal ions. (e) ATPase assay in the presence of 1 mM excess cold NTPs or dNTPs. Each vertical bar represents the % activity in the presence of γ-P^{32} ATP (bar-control) or cold NTPs (bars 3–6) or dNTPs (bars 7–10).

2a, therefore appears to be a strategy operating to regulate the activities of the domains.

An analysis of the P10 sequence showed that it does not contain the canonical NTP binding motifs, suggesting that deviant motifs could be employed for ATP hydrolysis. Viral ATPases like bacteriophage terminases, VP6 from *Blue tongue virus* and cellular ATPase MinD are also known to hydrolyze ATP in the absence of canonical NTP binding motifs [14,17,18].

Thus, the present studies demonstrate for the first time that P10 domain of SeMV polyprotein 2a has a novel ATPase activity which is influenced by the nucleic acid binding P8 domain. Both these domains might therefore, play essential roles in SeMV life cycle.

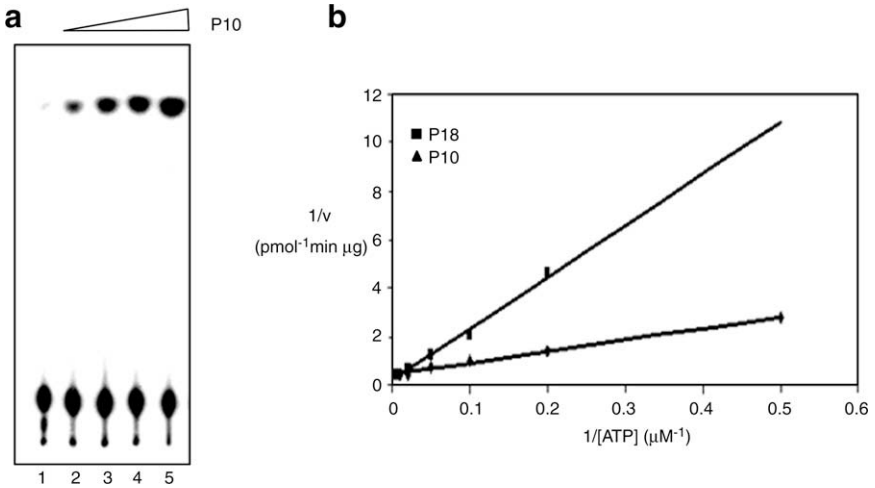

Fig. 5. (a) ATPase assay was performed with 0, 0.1, 0.2, 0.4, 0.8 μg (lanes 1–5) of P10 protein. (b) $1/v$ vs 1/[ATP], Lineweaver–Burk plot for P10 and P18 proteins. ATPase assays were performed with 0.35 μg P10 and P18 at increasing ATP concentrations (2, 5, 10, 20, 50, 100, 200, 500 μM) and the activity v was calculated.

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