Reconstitution of the RNA-dependent RNA polymerase activity of *Antheraea mylitta* cypovirus in vitro using separately expressed different functional domains of the enzyme

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*Antheraea mylitta* cytoplasmic polyhedrosis virus is a segmented dsRNA virus of the family *Reoviridae*. Segment 2 (S2)-encoded RNA-dependent RNA polymerase (RdRp) helps the virus to propagate its genome in the host cell of the silkworm, *Antheraea mylitta*. Cloning, expression, purification and functional analysis of individual domains of RdRp have demonstrated that the purified domains interact in vitro. The central polymerase domain (PD) shows nucleotide binding properties, but neither the N-terminal domain (NTD) nor the C-terminal domain (CTD). Isolated PD does not exhibit RdRp activity but this activity can be reconstituted when all three domains are included in the reaction mixture. Molecular dynamics simulation suggests that the isolated PD has increased internal motions in comparison to when it is associated with the NTD and CTD. The motions of the separated PD may lead to the formation of a less accessible RNA template-binding channel and, thus, impair RdRp activity.

**INTRODUCTION**

*Antheraea mylitta* cytoplasmic polyhedrosis virus (AmCPV) is a segmented dsRNA virus with 11 segments (S1–S11) as its genome. All these genomic segments have been cloned, sequenced and characterized at the molecular level (Qanungo et al., 2000, 2002; Sinha-Dutta et al., 2005; Jangam et al., 2006; Chavali & Ghosh, 2007; Chavali et al., 2008; Chakrabarti et al., 2010; Ghori et al., 2010; Biswas et al., 2014a, b). AmCPV is a member of the genus *Cypovirus* of the family *Reoviridae*, infecting Indian non-mulberry tasar silkworm *Antheraea mylitta*, and causes substantial economic loss each year (Jolly et al., 1974; Mertens et al., 2005). The mechanism of genomic RNA synthesis has not been studied in detail for any cytoplasmic polyhedrosis viruses (CPVs). Recently, a model for dsRNA synthesis has been postulated from the cryo-EM (Cryo-Electron Microscopy) polymerase map of *Bombyx mori* CPV (BmCPV-1) (Liu & Cheng, 2015; Zhang et al., 2015). Hence, a characterization of the RNA replication/translation process mediated by cyoviral polymerases is urgently required. AmCPV S2 encodes a 1116 aa-long (~123 kDa) RNA-dependent RNA polymerase (RdRp) that mediates transcription/replication of all the viral genome segments (Ghorai et al., 2010). No X-ray crystal structure is available for this RdRp, but a computational approach has deciphered its structure–function relationship to some extent (Kundu et al., 2015). It was found that the overall model structure of AmCPV RdRp is relevant to the structures of transcriptionally active BmCPV-1 RdRp (PDB 3JB7) (Zhang et al., 2015), orthoreovirus A3 (PDB 1N1H) (Tao et al., 2002), rotavirus VP1 (PDB 2R7O) (Lu et al., 2008) and the model structure of bluetongue virus (BTV) VP1 (Wehrfritz et al., 2007). Refinement of the reported model of AmCPV RdRp based on the coordinates of BmCPV-1 RdRp (PDB 3JB6 3JB7) has improved the quality of the model and precisely redefined the boundaries of the N-terminal domain (NTD) (residues 1–349), the polymerase domain (PD) (residues 350–879) and the C-terminal domain (CTD) (residues 880–1116) (Fig. 1). The domain organization of AmCPV RdRp is found to be different from that of the model structure of BTV polymerase, having an overlap of PD and CTD and also an unmodelled region between its NTD and PD. The unique domain organization of BTV polymerase may help it to interact with the other proteins residing in the BTV subcore (Wehrfritz et al., 2007).

The structure of the central PD of AmCPV RdRp can be compared to other viral polymerases with respect to the...
basic right-handed structure having a palm (residues 520–563, 641–735), thumb (residues 736–879) and fingers (residues 350–519, 564–640) subdomains. The presence of a large NTD and CTD is unique to members of the family Reoviridae. The NTD serves to anchor the fingertips to the thumb, the bracelet-like structure of the CTD acts as an exit route for the nascent dsRNA and both provide a large surface area to interact with other viral proteins (McDonald et al., 2009). It is not clear why members of the family Reoviridae possess bulky RdRps compared to other viral polymerases. For instance, RdRps of hepatitis C virus (HCV) (PDB 1QUV), rabbit haemorrhagic disease virus (PDB 1KHV), polio virus (PDB 1RDR) and bacteriophage φ6 (PDB 1HI0) contain 572, 516, 461 and 664 aa, respectively, and their structures closely resemble the PD of the Reoviridae RdRps (PDB 1MUK, 2R7O, 3JB7). Thus, the presence of the NTD and CTD is considered an appendage to the PD of the Reoviridae polymerases. It is hypothesized for the orthoreovirus A3 that NTD and CTD together sandwich the central PD to prevent its movement during catalysis (Tao et al., 2002), but this needs further illustration. It has also been found that the PD of BTV alone cannot synthesize a new RNA strand from the template RNA, but the RdRp activity can be reconstituted when the other purified individual domains (NTD and CTD) are mixed together (Wehrfritz et al., 2007). Since no work has been reported for any cytopviral RdRps to correlate internal motion of the enzyme to its activity, an elaborate understanding is required of the activity reconstitution process by the domains of CPV RdRps. Here, we report the cloning, overexpression and purification of different domains of AmCPV RdRp in the soluble form as fusion proteins in Escherichia coli. Using the fluorescence resonance energy transfer (FRET) approach, we show the ability of the different domains to interact with each other under in vitro conditions. Finally, we demonstrate that the PD can bind nucleotide, but alone cannot perform the RdRp activity. This activity can be reconstituted by mixing all three fusion tag-free domains, and this is confirmed by molecular dynamics (MD) simulation.

RESULTS

Expression and purification of the AmCPV RdRp domains and the full-length protein

Expression of AmCPV RdRp domains as NTD (residues 1–349), PD (residues 350–879) and CTD (residues 880–1116) failed to produce soluble protein (data not shown); instead the regions spanning 1–305, 307–857 and 858–1116 as NTD1 (~33 kDa), PD1 (~61 kDa) and CTD1 (~29 kDa) domains, respectively (Fig. 2a) were overexpressed in soluble form in E. coli BL21(DE3) cells by pCOLD-TF expression vector (TaKaRa). All these expressed proteins contained a His6-tag-trigger factor (TF) fusion tag (~55 kDa) at their N-terminal ends. Therefore, NTD1-TF (~88 kDa), PD1-TF (~116 kDa) and CTD1-TF (~84 kDa) were purified to homogeneity by metal affinity (Ni-NTA) chromatography. Moreover, PD1 with active site mutations in two aspartic acid residues, which were changed to alanine (D682A and D683A), was also expressed as a His6-TF fusion protein and purified. Full-length AmCPV RdRp (P123) was expressed in soluble form as a His6-tagged fusion protein in High Five cells via the baculovirus recombinant expression system and purified by Ni-NTA chromatography. The purity of the expressed proteins was analyzed by SDS-PAGE (Fig. 2b).

Interaction between individually expressed domains of AmCPV RdRp

To assess the interaction of the individually expressed and purified domains of AmCPV RdRp with each other in the absence of any other factor (e.g. RNA), steady state FRET experiments were conducted. Interference of the fusion tag present at the N-terminal end of the domains was minimized in the interaction assay by choosing two domains as
a FRET pair from the AmCPV RdRp model, in relation to which the N-termini are distantly located. Accordingly, NTD1-TF and PD1-TF were chosen as one FRET pair, while the other pair comprised PD1-TF and CTD1-TF. FITC and rhodamine isothiocyanate (RITC) were taken as donor and acceptor fluorescent molecules, respectively. In the FRET analysis between NTD1-TF and PD1-TF, the concentration of FITC-labelled NTD1-TF was set to 1 µM and the concentration of RITC-labelled PD1-TF was varied gradually from 0 µM to a higher concentration until the fluorescence emission maxima at 580 nm of the RITC-labelled protein reached saturation, at an excitation wavelength of 495 nm. For the pair PD1-TF and CTD1-TF, the concentration of FITC-tagged PD1-TF was set to 1.8 µM and the concentration of RITC-tagged CTD1-TF was increased gradually to saturation. Upon interaction between the members of a FRET pair, the fluorescence energy is transferred from the donor to the acceptor molecule and, thus, the emission intensity of the acceptor molecule increases while for the donor it decreases (Fig. 3a, c). A hyperbolic increase in the fluorescence emission intensity of the acceptor molecule(s) was observed in comparison with the control FRET experiments, indicating an interaction between the members of the pair. To measure the non-specific interactions (as a control), FITC-labelled TF was titrated with RITC-labelled NTD1-TF or PD1-TF or CTD1-TF, where non-specific interactions led to a linear increase/decrease in 580 nm emissions (Fig. S1, available in the online Supplementary Material). The same titration into buffer only (without FITC) gave a measure of the emission of the RITC-labelled proteins upon direct excitation at 495 nm, and was also found to be linear. This indicated that the nonspecific interactions were negligible. After subtracting the appropriate buffer control, NTD1-TF/PD1-TF and PD1-TF/CTD1-TF pairs showed approximate dissociation constants ($K_d$) of 0.017 (±0.0047) µM and 0.013 (±0.0025) µM, respectively, as obtained from binding isotherm fitted to the Hill equation (see Methods; Fig. 3b, d). Additionally, for both pairs, a hypochromic blue shift in the donor fluorescence peak was observed with increasing concentration of the acceptor molecule. The fluorophore that displays a blue shift (peak shift towards the lower wavelength) in the emission spectrum has higher quantum yield or life-time, which is the same as when the fluorophore is placed in a less polar solvent (Lakowicz, 2006). Thus, the shift is attributed to the changing micro-environment of the donor molecule from polar to a non-polar environment, i.e. hydrophobic forces are expected to guide inter-domain interaction.
Nucleotide binding assay

N-Methyl-3'-O-anthranoyl-GTP (mant-GTP) was used to determine the binding of a nucleotide with different AmCPV RdRp domains, and the full-length polymerase (P123) was used as a positive control. Mant-GTP is a fluorescent guanine nucleotide analogue widely used to characterize nucleotide binding proteins, and has an advantage over using radioactive nucleotide in terms of ease of detection and handling (Sullivan et al., 2000). Changes in mant fluorescence reflect the hydrophobic environment of the nucleotide analog. The change in fluorescence of the guanine nucleotide analogue was monitored as an indication of protein–mant-GTP binding. Fig. 4 shows typical excitation spectra from mant-GTP alone and those bound to protein monitored at an emission wavelength of 446 nm. The relative fluorescence intensity increased significantly (almost twofold) when mant-GTP was added to either wild-type PD1-TF or P123 compared to the fluorescence signal of mant-GTP alone at an excitation wavelength of 361 nm. In contrast, NTD1-TF, CTD1-TF and the fusion tag TF per se did not show any increase in fluorescence intensity upon mixing with mant-GTP. These results indicate that the nucleotide-binding property of full-length AmCPV RdRp was retained with the isolated PD even in the absence of the other two domains. An experiment performed with the active site mutant of PD1-TF did not show any mant-GTP
binding, suggesting that nucleotide binding occurs in the active site of the PD.

**Reconstitution of AmCPV RdRp activity from the isolated domains**

To determine whether the PD was sufficient to show RdRp activity, an assay was done using the 3’ end (185 bases) of AmCPV S2(+) RNA as a template. When PD1-TF alone was tested, no discrete product band formation was observed. This activity could not be restored either when PD1-TF was mixed with either NTD1-TF or CTD1-TF or both (data not shown). The fusion tag TF was then cleaved from the N-terminus of PD1 (Fig. 5a, lane 3) using purified His6-HRV-3c protease (Fig. 5a, lane 1) and subsequently purified (partially) via subtractive Ni-NTA chromatography (Fig. 5b, lane 2). Then, the tag-free PD1 alone (Fig. 5c, lane 3) and in combination with either NTD1-TF or CTD1-TF or both were subjected to RdRp assay, but no product RNA was obtained (data not shown). Investigation through the model structure of AmCPV RdRp has revealed that the N-terminal ends of both CTD1 and the NTD1 are close enough to hinder proper inter-domain organization when both contain a bulky fusion tag at their N-termini. Therefore, NTD1-TF and CTD1-TF were also subjected to HRV-3c protease digestion (Fig. 5a, lanes 2 and 4) as described above, and purified partially as tag-free NTD1 and CTD1 (Fig. 5b, lanes 1 and 3). After mixing the three tag-free domains in all possible combinations (keeping PD1 in common in each case) and testing for RdRp activity in the presence of [α-32P]UTP, it was observed that only when all three domains were combined did [32P]-labelled product RNA synthesis occur from the RNA template (Fig. 5c, lane 5), indicating restoration of enzymatic activity that is comparable to the activity of full-length polymerase (P123) (Fig. 5c, lane 1). The tag-free NTD1 and CTD1 domains were not tested separately for enzyme activity, because it has previously been reported that the NTD and CTD of the Reoviridae polymerase cannot individually perform the RdRp function (Wehrfritz et al., 2007). Under denaturing conditions, the [32P]-labelled product RNA migrates the same distance as that of the template RNA (Fig. 5d, lanes 1 and 4), indicating formation of the template-sized product strand. The [32P]-labelled product RNA was formed only in the presence of all four nucleotides along with [α-32P]UTP in the assay mix, which excludes the possibility of forming [32P]-labelled RNA following the addition of [α-32P]UTP to the 3’ end of the template RNA by terminal transferase activity (lane 4) of AmCPV RdRp. Moreover, the product RNA generated via RdRp activity was treated with RNase A under high (300 mM) and low salt (50 mM) concentrations (Fig. 5e). RNase A is known to digest both ssRNA and dsRNA at low salt concentrations, while it cannot digest dsRNA at high salt concentrations. In our experiment, no [32P]-labelled RNA band was observed in the gel when the product RNA was digested with RNase A at a low salt concentration (lane 1); however, a [32P]-labelled product RNA band was found when the product RNA was digested with RNase A at high salt concentrations (lane 3), indicating that most of the products were double-stranded by forming a stable duplex with the template.

**Isolated PD has a flexible thumb**

Our proposed model structure of AmCPV RdRp (Kundu et al., 2015) has been refined based on the cryo-EM structures of the quiescent and transcriptionally active forms of BmCPV-1 RdRp (PDB 3JB6 and 3JB7). Resulting AmCPV polymerase models in the quiescent and active forms differ significantly in regard to their CTD (Figs 1 and S2). The isolated PD models from both forms of RdRp and the full-length models were subjected to MD simulation. This was found that dynamics of both forms do not differ by much (Fig. S3), but a comparison of the dynamic properties between PD per se and as a part of the full-length RdRp gave interesting results. For clarity, only the transcriptionally active form of the models could be considered. Following MD simulation for 10 ns (or 10 000 ps), the three-dimensional structures of the PD and the full-length protein had reached equilibrium and root mean square deviations (RMSD) of the Cα atoms converged to almost 6 and 3.5 Å for the domain and full-length protein, respectively (Fig. 6a), indicating higher structural flexibility of the separate PD over the full-length RdRp. To highlight the difference, a comparative root mean square fluctuation...
(RMSF) plot was made between the two (Fig. 6b), showing that the thumb (residues 736–879) exhibits significantly increased fluctuation for the isolated domain. The thumb interacts with the fingers, making a closed right-handed structure of the PD. This interaction leaves sufficient space between the thumb and fingers subdomains so that the RNA template can pass through it into the reaction centre.

During simulations, the PD as part of the full-length enzyme remained similar to the active closed conformation, while the isolated domain simulation underwent a marked conformational change in which the thumb subdomain reoriented into a conformation where parts of it moved much closer to the fingers subdomain (Fig. 6c). Cumulative solvent-accessible surface area (SASA) values of the finger

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**Fig. 5.** Reconstitution of AmCPV RdRp activity. (a) 10 % SDS-PAGE analysis of various HRV-3c-protease-digested AmCPV RdRp domains fused with TF tag. Lane 1, purified His$_6$-HRV-3c protease; lanes 2, 3 and 4, protease cleavage products of NTD1-TF, PD1-TF and CTD1-TF, respectively. (b) 10 % SDS-PAGE analysis of the flow-through protein from subtractive NiNTA chromatography of 3c-protease-digested AmCPV RdRp domains. Lanes 1, 2, 3 and 4 show TF tag-free NTD1, PD1, CTD1 and 3c-protease-treated TF fusion tag, respectively. Partially purified tag-free domains (indicated by arrows) contain fractions of undigested (filled star) and fusion proteins (blank star) as purification artefacts. M, protein molecular mass (kDa) marker. (c) Upper panel, autoradiograph of the $^{32}$P-labelled RdRp reaction products electrophoresed in 7 M urea-5 % PAGE gels. Tag-free PD1 alone (lane 3) or in combination with tag-free NTD1 (lane 2) or CTD1 (lane 6) or all three together (lane 5) was tested for AmCPV RdRp activity. During the assay, purified P$_{123}$ (lane 1) was used as a positive control, and no protein (lane 4) was used as a negative control. The lower panel represents the incorporation of radio-labelled nucleotide (in c. p.m.) in the reactions of the respective lanes in the upper panel, indicating maximal incorporation in lanes 1 and 5 (red colored text in the lower panel) for the synthesis of $^{32}$P-labelled RNA, with the band indicated by the arrow. (d) Autoradiograph of 7M urea-5 % PAGE gel showing electrophoresed RdRp reaction products in the presence of [α-$^{32}$P]UTP alone (lane 2) or in association with only UTP (lane 3) or all four nucleotides (lane 4) indicating formation of the $^{32}$P-labelled product RNA band (indicated by an arrow) only in the presence of all four nucleotides. The size of the product is shown equivalent to the size of the $^{32}$P-labelled RNA template (lane 1) under denaturing conditions. (e) Analysis of RNase A-treated $^{32}$P-labelled RdRp product under high salt (300 mM NaCl) (lane 3) and low salt (50 mM NaCl) (lane 1); lane 2 represents the $^{32}$P-labelled RNA product not treated with RNase A and the slot between lane 1 and lane 2 is empty. The inclusion or omissions are indicated by + or −, respectively.
residues (T431, R433 and S434) residing in the template entry channel (Kundu et al., 2015) were calculated in the mean structures after simulations. Cumulative SASA of these residues was calculated to be 265 Å² for the full-length enzyme, while it was reduced for the isolated PD to 151 Å². The lower SASA in the template channel of the separate domain reflects a close association between the fingers and thumb subdomains (Fig. 7a, b), which mitigates against RNA substrate entry into the template channel. Thus, it is logical to assume that PD, in association with NTD and CTD, i.e. in the intact full-length form, has restricted thumb flexibility that facilitates RNA template entry into the active site and can restore the activity of the full-length AmCPV polymerase. This is consistent with our experimental evidence that RdRp activity is not associated with isolated PD, while the involvement of NTD and CTD is also required to re-establish the activity of the enzyme.

DISCUSSION

Different domains of AmCPV RdRp were expressed in E. coli with or without fusion tag, and the proteins expressed were able to maintain their solubility in the absence of the fusion tag. In contrast to BTV PDs that have been isolated as soluble proteins from bacteria (Wehrfritz et al., 2007), AmCPV RdRp domains required a fusion tag to isolate them as soluble proteins from bacteria. However, these protein domains expressed remain soluble upon subsequent
removal of the tag. Purified individual domains of AmCPV RdRp can interact in vitro, implying that specific surface contacts are more important for interaction between the domains irrespective of whether the polypeptide backbone is continuous or not. Moreover, this observation has excluded the possibility that RNA is an essential factor in bringing the domains into the interaction milieu. In the case of full-length AmCPV RdRp, the involvement of three domains may be indispensable for the recognition of virus-specific RNA. However, the PD of AmCPV RdRp can behave like the full-length protein in terms of binding with nucleotide by two active site aspartic acid residues, and this is consistent with the earlier observation of Wehrfritz et al. (2007) that the central PD of BTV polymerase can bind radio-labelled nucleotide. In contrast, recovery of RdRp activity by the isolated domains of AmCPV polymerase requires removal of the N-terminal fusion tag, followed by mixing of all three domains in the assay system, and this activity produces dsRNA by forming duplex with the template. This is not surprising, since the RNA template used was vastly excessive relative to the RNA products. Our observation is consistent with the in vitro RNA synthesis by recombinant HCV RdRp (Oh et al., 1999) and the BTV polymerase (Wehrfritz et al., 2007). Notably, HRV 3c protease digestion leaves extra amino acid residues at the N- and C-termini of the domains as cloning artefacts, which are likely to have a minimal effect on inter-domain interaction and restoration of polymerase activity (Fig. S4). Furthermore, fractions of the undigested domains and the fusion tag regarded as purification artefacts in the tag-free NTD1, PD1 and CTD1 domains do not seem to have had significant influence on the reconstitution of polymerase activity. Furthermore, reconstitution of this activity by the individual domains indicates proper folding of the domains.

Concerted movement of thumb and fingers subdomains with respect to palm is biologically relevant to the function of right-handed RdRps in terms of making a closed form of the active site favourable for nucleotidyl transfer reaction. Perturbations to motion lead to the inactive form of the enzyme (Liu et al., 2006; Ivetac & McCammon, 2009; Moustafa et al., 2011). In contrast to these polymerases, for RdRps of the family Reoviridae there are very limited data on their innate motions. Recent cryo-EM maps of BmCPV-1 virions in quiescent and transcriptionally active states have suggested a significant conformational change in the CTD of BmCPV-1 RdRp located inside the virion core (Zhang et al., 2015). However, MD simulation cannot capture such transitions in the model of AmCPV RdRp either in the quiescent or in the active form. Thus, conformational change in RdRp CTD is apparently influenced by the other proteins and dsRNAs inside the CPV virion core in an environment favourable for transcription. Surprisingly, altered thumb dynamics of the isolated PD in comparison to when it is a part of the full-length form has led to the formation of a more closed structure over time. The closed form is relevant to the ‘hyper-closed’ form of the HCV polymerase NS5B in the presence of a non-nucleoside inhibitor, which acts as a ‘molecular staple’ to stitch the thumb in an altered conformation (Davis & Thorpe, 2013). Both structures bear narrower RNA template grooves by having a close association of the thumb with the fingers subdomain. This structural fault is likely to block RNA template grooves by having a close association of the thumb with the fingers subdomain. This structural fault is likely to block RNA template entry into the active site of the isolated PD, and thereby impair RdRp activity. Restoring AmCPV RdRp activity by the association of three domains together implies that NTD and CTD act together to restrict movement of the polymerase thumb, so that an active conformation of the enzyme is created. In other words, the isolated domains must function together or in concert to re-establish the activity of the full-length RdRp. Apart from the other functions of NTD and CTD as described elsewhere (McDonald et al., 2009), a new functional aspect of these domains has been established for RdRps of the family Reoviridae in the light of AmCPV
polymers. We believe that our experimental data, along with bioinformatics analysis, will not only foster the development of a comprehensive description of the cypoviral replication/transcription process, but also set a stage to re-evaluate the structure–function relationship of the polymers of the family Reoviridae. It should also be noted that some of the RdRps of the Reoviridae require additional viral core proteins to exert polymerase activity in vitro (McDonald & Patton, 2009; McDonald et al., 2009).

**METHODS**

**Cloning, protein expression and purification.** Nucleotide sequences encompassing NTD1, PD1 and CTD1 of AmCPV RdRp were PCR amplified from the plasmid harbouring AmCPV S2 ORF construct (Ghorai et al., 2010) using respective forward (F) and reverse (R) primers (Table 1) as depicted in Fig. 1(a), while the internal primers used for PD1 mutation (D682A and D683A) were PD1_mF1 and PD1_mR2 (Table 1). The PCR amplicons were cloned in pCOLD-TF expression vector (TaKaRa) and expressed according to the manufacturer’s protocol. The proteins were purified by Ni-NTA chromatography and finally stored in buffer A containing 5 mM Tris-HCl pH 8.0, 30 mM NaCl and 10 % glycerol (v/v).

In order to obtain full-length AmCPV RdRp (P123), recombinant baculoviruses containing the AmCPV S2 ORF construct (Ghorai et al., 2010) were allowed to infect actively grown High Five cells (Life Technologies) (0.6 × 10^6 cells ml^{-1}) at an m.o.i. of ~ 30 and incubated at 23 °C. At 76 h post-infection, cells were harvested and purified P123, was obtained as described by Ghorai et al. (2010). Purified P123 was finally stored in buffer containing 25 mM HEPES pH 7.5, 100 mM KCl, 50 mM NaCl and 10 % glycerol (v/v).

His6-HRV-3c protease (~22kDa) expressed in *E. coli* BL21(DE3) cells harbouring the PET 24d (+)-HRV-3c protease construct (a kind gift from the EMBL Hamburg, Hamburg, Germany) was purified by Ni-NTA chromatography and finally stored in buffer containing 10 mM Tris-HCl pH 8.0, 300 mM NaCl, 0.5 mM EDTA and 10 % glycerol (v/v).

The protein concentrations were determined by the Bradford method (using BSA as the standard) (Bradford, 1976) and stored in small aliquots in –80 °C. Purity of all proteins was analysed by standard 10 % SDS-PAGE.

**Protease cleavage and subtractive Ni-NTA chromatography.** In order to remove the N-terminal fusion tag (His6-TF) from the domains, each of the purified domains, NTD1-TF, PD1-TF and CTD1-TF, was mixed with purified His6-HRV-3c protease separately at a molar ratio of 1:20 (protease : protein) in a buffer containing 50 mM Tris-HCl, 500 mM NaCl, 0.5 mM EDTA (pH 8.0) and incubated for 16 h at 4 °C.

Then, the mixtures were separately loaded onto a Ni-NTA column pre-equilibrated with the same buffer containing 1 M urea. Protease-cleaved domains devoid of the His6-TF fusion tag were collected in the flow-through fraction. The majority of the cleaved fusion tag, along with the undigested proteins and the His6-tagged HRV-3c protease, remained bound to the Ni-NTA column. Proteins present in the flow-through portions were further dialysed against buffer A at 4 °C for overnight to remove the urea, and stored in small aliquots at –80 °C.

**FRET experiment.** To analyse the interactions of the isolated domains of AmCPV RdRp under in vitro conditions, FRET experiments were carried out with fluorescent-labelled domains at 25 °C. For this, purified domains were tagged with either FITC or RITC dyes (Sigma) at a molar ratio of 1:100 (protein : dye) following the manufacturer's protocol. After removal of the free dyes, concentration of dye-bound protein was measured by the equation: 

$$[\text{Protein}] = \left(\frac{A_{280} - (A_{280} \times \text{dilution factor})}{\varepsilon \times \text{dilution factor}}\right) \\ = \left(\frac{A_{280} - (A_{280} \times \text{dilution factor})}{\varepsilon \times \text{dilution factor}}\right),$$

wherein acceptor molecule’s fluorescence intensity, maximum fluorescence intensity and minimum fluorescence intensity are designated as $F$, $F_{\text{max}}$ and $F_{\text{min}}$, respectively; symbol $\varepsilon$ denotes protein molar absorption coefficient ($\text{M}^{-1} \text{cm}^{-1}$); $C$ is the correction factor for the absorbance of dye at the wavelength of 280 nm. The FITC-tagged protein as the fluorescent donor molecule was titrated by RITC-tagged protein as the fluorescent acceptor molecule using a Jobin Horiba FluoroMax 4 spectrofluorometer. The excitation wavelength was kept at 495 nm and the emission spectra were scanned from 500 to 650 nm. An integration time of 0.5 s was used and excitation or emission monochromators were set to 2 and 4 nm for the NTD1-TF/PD1-TF pair and the PD1-TF/CTD1-TF pair, respectively. Spectra were taken by subtracting the scan from the appropriate buffer blank. Data obtained from the experiment were fitted to the Hill equation:

$$F = F_{\text{max}} + \left(\frac{F_{\text{max}} - F_{\text{min}}}{(K_d + [\text{S}])}\right),$$

wherein $K_d$ represents the dissociation constant.

**Nucleotide binding assay.** Binding of protein to mant-GTP, a fluorescent nucleotide analogue, was measured by Jobin Horiba FluoroMax 4 spectrophotometer at 25 °C and indicated by the increase in fluorescence of the mant analogues in the presence of the protein. During the assay, 100 nM mant-GTP in binding buffer B (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM MgCl2, 50 nM Zn(OAc)2) was mixed with 0.5 µM of various RdRp domains or the full-length proteins and subjected to fluorescence excitation measurement at a fixed emission wavelength of 446 nm, while excitation spectra were recorded from 310 to 410 nm. Appropriate buffer blank was analysed during the recording of each of the spectra, and relative fluorescence intensity was calculated as the fluorescence signal of either the free or protein-bound mant nucleotide at any excitation wavelength, divided by the fluorescence signal of free mant nucleotide at an excitation wavelength of 361 nm.

**RdRp assay.** The RdRp assay was performed as described by Ghorai et al. (2010) with some minor modifications. For this, ssRNA substrate (185 bases) was prepared by in vitro transcription from the 3’ end of AmCPV S2 (+) strand RNA as minigenome using a MaxiScript Kit (Ambion). The transcript was rendered devoid of the DNA template by 2 U Turbo DNase (Ambion). Finally the transcript was purified by RNase free minilute kit (Qiagen) and quantified by NanoDrop spectrophotometer (Thermo Scientific). Almost 1 µg of purified RNA transcript was incubated in 50 µl reaction mixture containing 1 µ buffer B, 5 mM KCl, 5 mM DTT, 1 % purified BSA, 1 mM ribonucleotide mix, 50 µg actinomycin D ml^{-1}, 20 U RNase inhibitor (NEB) and 2 µ (32 P)UTP [specific activity 3500 Ci mmol^{-1} (129.5 TBq mmol^{-1})] (BRIT, India) and 1 µg full-length purified RdRp or 0.5 to 1.5 µg of the domains at 32

**Table 1. Sequences of the primers used to clone the domains of AmCPV RdRp**

<table>
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<th>Primer name</th>
<th>Sequence</th>
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<tr>
<td>202F</td>
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</tr>
<tr>
<td>204R</td>
<td>5'-TAAAGCTCCAGATGTACCTTGAAAATAC-3'</td>
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<td>198F</td>
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<tr>
<td>PD1_mF1</td>
<td>5'-GAAATGCGTCCGCTGCTGCTATCACAAGG-3'</td>
</tr>
<tr>
<td>PD1_mR2</td>
<td>5'-TCTTGTTGATAGCAGCCGAGCACATTC-3'</td>
</tr>
</tbody>
</table>

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ACKNOWLEDGEMENTS

This work was supported by a grant from the Department of Science and Technology, Government of India (no. SR/SO/BB-0038/2011). A. K., M. B. and A. R. C. acknowledge IIT Kharagpur, CSIR (GOI) and UGC (GOI), respectively, for research fellowships. The authors also thank Dr Agneyo Ganguly for helping with the fluorescent study and Mr Anirudha Dutta for helping with the MD simulation.

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