Phosphoprotein of the Rinderpest Virus Forms a Tetramer through a Coiled Coil Region Important for Biological Function

A STRUCTURAL INSIGHT*

Abdur Rahaman‡§, Naryanaswamy Srinivasan¶, Narayanashwamy Shamala**, and Melkote Subbarao Shaila‡ ‡‡

From the ‡Department of Microbiology and Cell Biology, the ¶Molecular Biophysics Unit, and the **Department of Physics, Indian Institute of Science, Bangalore 560012, India

Phosphoprotein (P) of negative sense RNA viruses functions as a transcriptional transactivator of the viral polymerase (L). We report here the characterization of oligomeric P protein of rinderpest virus (RPV) and provide a structural basis for its multimerization. By size exclusion chromatography and dynamic light scattering analyses we show that bacterially expressed P protein exists as an oligomer, thus excluding the role of phosphorylation in P protein oligomerization. Gel filtration analyses of various parts of the P protein, also expressed in Escherichia coli, revealed that the predicted coiled coil region in the C-terminal domain is responsible for P protein oligomerization. Dynamic light scattering analysis confirmed the oligomeric nature of the coiled coil region of P. Chemical cross-linking analysis suggested that the C-terminal coiled coil region exists as a tetramer. The tetramer is formed by coiled coil interaction as shown by circular dichroism spectral analysis. Based on sequence homology, we propose a three-dimensional structure of the multimerization domain of RPV P using the crystal structure for multimerization domain of sendai virus (SeV) P as a template. Four-stranded coiled coil structure of the model is stabilized by a series of interactions predominantly between short nonpolar side chains emerging from different strands. In an in vivo replication/transcription system using a synthetic minigenome of RPV, we show that multimerization is essential for P protein function(s), and the multimerization domain is highly conserved between two morbilliviruses namely RPV and pest of small ruminants virus. These results are discussed in the context of biological functions of P protein among various negative-stranded RNA viruses.

Rinderpest virus (RPV),† which causes rinderpest disease in large and small ruminants is an enveloped virus belonging to the morbillivirus genus of the family Paramyxoviridae. The negative sense, single-stranded RNA genome codes for six structural proteins: namely nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin protein (H), and large protein (L). The viral N-RNA i.e. genomic RNA encapsidated with N protein is transcribed and replicated by the L (RNA-dependent RNA polymerase) and P complex (1). The L protein is associated with N-RNA template through its interaction with P protein to form the transcribing ribonucleoprotein (RNP) complex. In addition to polymerization activity, L exhibits a number of other enzymatic activities including methyl transferase, 5'-cap synthesis of mRNA, and poly(A)⁺ polymerase (2). During transcription, the intergenic start/stop signals are recognized by polymerase complex resulting in the synthesis of monocistronic, capped, and polyadenylated mRNAs. Once the intracellular concentration of viral proteins reaches a threshold level, genome replication begins. The intracellular concentration of unassembled N protein (N₀) is believed to regulate the switch from transcription to replication (3). During replication, the same polymerase complex ignores stop signals and generates full-length unmodified encapsidated antigenomic RNA to serve as the template for the synthesis of progeny viral genomes.

P proteins of negative-stranded RNA viruses play multiple roles during viral infection. They act as a transcriptional transactivator and recruit L protein onto viral N-RNA template (1, 4). P proteins also bind to the N-RNA template, independent of its role in the L-P polymerase complex, and activate transcription (5). In addition to binding with the assembled nucleocapsid structure of the N-RNA template, P proteins interact with unassembled N proteins and prevent nonspecific aggregation of the latter by forming the N₀-P complex, a precursor for encapsidating newly synthesized RNA during replication (6). P proteins of mononegalovirales undergo phosphorylation in one or more serine residues, which has been shown to be important for its function (7, 8). Although P proteins function as a homooligomer, their oligomerization status as well as the requirement of phosphorylation for oligomerization has been shown to vary among them (1, 9–17). P proteins of all the paramyxoviruses harbor a coiled coil region at the C-terminal domain, and this region has been shown to be important for oligomerization in a number of viruses in the Paramyxoviridae family (13, 18–21). The P protein has a modular structure, which comprises two major domains: the N-terminal domain is highly acetic acid; PCT, P C-terminal region; FNT, P N-terminal region; CAT, chloramphenicol acetyltransferase; SEC, size exclusion chromatogra- phy; SeV, sendai virus; PPRV, pest of small ruminants virus; DLS, dynamic light scattering.

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† A senior research fellow of the University Grant Commission, Government of India.
‡ Supported by the Senior Research Fellowship program for Biomedical Research by the Wellcome Trust, London.
§ A senior research fellow of the University Grant Commission, Government of India.
¶ To whom correspondence should be addressed: Dept. of Microbiology and Cell Biology, Indian Institute of Science, Bangalore 560012, India. Tel.: 91-80-23600139/22932702; Fax: 91-80-23602697; E-mail: shaila@mcbl.iisc.ernet.in.
** From the Department of Microbiology and Cell Biology, the Molecular Biophysics Unit, and the Department of Physics, Indian Institute of Science, Bangalore 560012, India.

This paper is available on line at http://www.jbc.org
variable among various paramyxoviruses whereas the C-terminal domain, though exhibiting low sequence similarity, is conserved in terms of secondary structure (13). The C terminus has been shown to have two subdomains in the sendai virus P protein: PMD, corresponding to the N-terminal region of the C-terminal domain that harbors the multimerization domain along with the L binding domain, and Px, corresponding to the rest of the C-terminal domain involved in nucleocapsid binding (13). Earlier work on the RPV P protein has shown that while the N terminus of the gene encodes 59 amino acid residues at the N terminus, a stop codon was incorporated in the reverse primer to eliminate additional amino acids at the C terminus.

Expression and Purification of Recombinant Proteins—E. coli BL21 (DE3) strain was transformed with plasmids carrying full-length as well as different parts of the RPV P. The transformant was grown in LB containing 100 µg/ml ampicillin (except for RPV P) or 50 µg/ml kanamycin (for RPV P) and induced with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside at an OD600 of 0.6 and grown for another 5 h. The cells were harvested and lysed by sonication in MCAC buffer (500 mM NaCl in 20 mM Tris-HCl, pH 8) and supplemented with 2 mM phenylmethylsulfonyl fluoride and protease inhibitor mixture. The lysates were centrifuged, and supernatant was mixed with Ni-NTA agarose. The resin was washed with 100 bed volumes of MCAC buffer containing 50 mM imidazole except for RPV P in which imidazole was not used. Proteins were then eluted with 500 mM imidazole in MCAC supplemented with a protease inhibitor mixture. The RPV P so obtained was dialyzed against 50 mM Tris-Cl, pH 8.0 and further purified by passing through a 5-ml Q-Sepharose column using 0–500 mM NaCl in dialysis buffer as the gradient. Eluted samples of purified proteins were detected by Coomassie Blue staining of SDS-polyacrylamide gels. The protein concentration was measured by taking absorbance measurements at 280 nm, except for RPV P, where the concentration was estimated by the Bradford assay. The identity of the proteins was confirmed by Western blot analysis using polyclonal antibodies raised in rabbit against bacterially expressed RPV P or PPRV P protein.

Size Exclusion Chromatography (SEC)—Either the Sephadex G75 column (45 cm × 2.22 cm, bed volume of 100 ml) or Sephacryl S300 column (60 cm × 2 cm, 120-ml bed volume) was equilibrated with PBS or MCAC buffer, respectively, and calibrated using standard protein molecular mass markers. One milligram each of P, PNT, and PCT in MCAC or RPV and Px in 1 ml of PBS were separated on Sepharose S300 or Sephadex G75, respectively, and the elution profiles were monitored by measuring the absorbance at 280 nm, except for RPV P, which was monitored by protein estimation using the Bradford assay. The proteins were then identified using SDS-polyacrylamide gels and silver staining.

Tetramerization of RPV P Is Important for Its Function

EXPERIMENTAL PROCEDURES

Materials—Escherichia coli DH5α strain was used for the maintenance of plasmids whereas the BL21 (DE3) strain was used for the expression of recombinant proteins (Invitrogen). pQE and p4a harboring the 1–291 (PNT) and 292–508 (PCT) amino acid regions of the P protein, respectively, were earlier cloned in the laboratory in pRSET vector (Invitrogen). p3e and p4a harboring the 1–291 and 292–508 amino acid residues at the C terminus. A stop codon was incorporated in the reverse primer to eliminate additional amino acids at the C terminus.

Expression and Purification of Recombinant Proteins—E. coli BL21 (DE3) strain was transformed with plasmids carrying full-length as well as different parts of the RPV P. The transformant was grown in LB containing 100 µg/ml ampicillin (except for RPV P) or 50 µg/ml kanamycin (for RPV P) and induced with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside at an OD600 of 0.6 and grown for another 5 h. The cells were harvested and lysed by sonication in MCAC buffer (500 mM NaCl in 20 mM Tris-HCl, pH 8) and supplemented with 2 mM phenylmethylsulfonyl fluoride and protease inhibitor mixture. The lysates were centrifuged, and supernatant was mixed with Ni-NTA agarose. The resin was washed with 100 bed volumes of MCAC buffer containing 50 mM imidazole except for RPV P in which imidazole was not used. Proteins were then eluted with 500 mM imidazole in MCAC supplemented with a protease inhibitor mixture. The RPV P so obtained was dialyzed against 50 mM Tris-Cl, pH 8.0 and further purified by passing through a 5-ml Q-Sepharose column using 0–500 mM NaCl in dialysis buffer as the gradient. Eluted samples of purified proteins were detected by Coomassie Blue staining of SDS-polyacrylamide gels. The protein concentration was measured by taking absorbance measurements at 280 nm, except for RPV P, where the concentration was estimated by the Bradford assay. The identity of the proteins was confirmed by Western blot analysis using polyclonal antibodies raised in rabbit against bacterially expressed RPV P or PPRV P protein.

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Dynamic Light Scattering (DLS) Analysis—About 1 mg/ml of RPV in (50 mM Tris-Cl, pH 8.0 and 50 mM NaCl) or 0.5 mg/ml of P (in 20 mM Tris-Cl, pH 8.0, and 500 mM NaCl) was subjected to DLS analysis using DynaPro software. The viscosity used for Rg calculation was estimated from the refractive index of the buffer as measured by refractometer.

Circular Dichroism (CD) Spectroscopy—Purified RPV at 0.1 mg/ml in PBS or in 50% trifluoroethanol in PBS was analyzed by a spectropolarimeter (JASCO J-715) at room temperature. The CD spectrum was measured in a cuvette of 2-mm path length, with a bandwidth of 0.5 nm and a scan speed of 50 nm/s. The buffer spectrum was subtracted from the protein spectrum. An average of four independent measurements were used to calculate molar residue ellipticity [θ]mrw using Equation 1,

\[ [\theta]_{mrw} = (\theta-100\text{-M},c\text{-V}_{N}) \]  

where [θ] is the mean residue molar ellipticity in deg cm² dmol⁻¹, M is experimental ellipticity in millidegree, c is protein concentration in mg/ml; l is cuvette path length in centimeters, and N is the number of residues of the protein. The percent helicity was estimated in Equation 2 (24, 25),

\[ \% \text{ helicity} = \frac{[\theta]_{222} - [\theta]_{198}}{[\theta]_{222}} \times 100 \]  

where [θ]222 is the experimentally observed absolute mean residue ellipticity at 222 nm and values for [θ]198 are corresponding to 198 nm, which were estimated at 32,000 and 2,000 deg²/cm²/dmols, respectively (25, 26).

In Vivo Replication/Transcription Assay—To assess the significance of tetramerization on the biological function of the P protein, an in vivo replication-transcription assay using the minigenome construct pMDB8A was performed as described earlier (23). The transcript from the minigenome is antigenomic sense, which is replicated to genomic sense RNA by the virus proteins, L, N, and P; expressed by co-transfected plasmids in A549 cells infected with recombinant vaccinia virus expressing T7 RNA polymerase. The newly made genomic RNA was then transcribed into CAT mRNA, and the translated CAT protein was...
measured by ELISA (Roche Applied Science). A549 cells (1 × 10^6 cells/55-mm dish) were infected with recombinant vaccinia virus, VTF7-3 at a multiplicity of infection of 10 at 37 °C. At 1-h postinfection, the cells were washed with PBS and transfected using 5 μl of LipofectAMINE (2 mg/ml) in 1 ml of OPTI-MEM medium (Invitrogen) containing 1 μg each of pMD6BSA, pKS-N, pRP6, and 100 ng of pGEM-L with or without pRPC/pPPMD. At 48-h post-transfection, the cells were harvested, and CAT activity was assayed by ELISA.

**Co-expression of Full-length RPV P with the PPRV P Multimerization Domain**—The plasmid DNA of pRP6 and pPPMD clones were co-transformed into BL21 (DE3) strains of E. coli, and the recombinant cells harboring both plasmids were selected using two antibiotics, i.e. ampicillin (100 μg/ml) and kanamycin (50 μg/ml). Transformed cells were grown in Luria Broth supplemented with 100 μg/ml of ampicillin and 50 μg/ml of kanamycin to an OD₆₀₀ of 0.6 at 37 °C. Expression and the purification of the protein by Ni-NTA agarose affinity chromatography were done as described above. The purity of both the purified proteins was tested by electrophoresis on a 15% SDS-polyacrylamide gel followed by Coomassie Blue staining and confirmed by Western blot analysis using the appropriate antibody.

**Prediction of Secondary Structures and Coiled Coil Regions**—The sequence of the multimerization domain of RPV P-protein (PMD) was subjected to secondary structure prediction analysis using PPHD as well as coiled coil region prediction (27–31). These predictions were employed in order to get views about the potential of this region to adopt α-helical coiled coil structure as well as to form coiled coils, independent of the fact that a distant homologue (Sendai virus phosphoprotein) has the same structural features.

**Comparative Modeling of the Coiled Coil Region of the P Protein**—The amino acid sequence of RPV FMD protein (amino acids 286–388) was aligned with that of the Sendai virus phosphoprotein whose crystal structure shows a homotetrameric α-helical coiled coil structure (21). The two proteins are distantly related, and the alignment is non-trivial. Hence the structural features (such as solvent accessibility and secondary structure) at every residue were evaluated, and relationships such as hydrogen-bonding patterns in the crystal structure were assessed. While aligning the sequences the probability of a residue in the RPV PMD protein adopting the structural environment of equivalent residues in the known structure was considered. The positive matches between predicted secondary structures in the P protein and the observed secondary structures in the crystal structure during alignment were also given importance.

The state of programs encoded in COMPOSER and incorporated in SYBYL (Tripos Inc., St. Louis) was used to generate a three-dimensional model of the P-protein (32). The COMPOSER-generated model was energy-minimized in SYBYL using the AMBER force field (33). The energy-minimized model of a subunit of RPV PMD was superimposed with each one of the four subunits of SeV PMD, and the preliminary model for RPV PMD tetramer, so obtained, was subjected to further energy minimization to optimize interprotomer interactions.

**RESULTS**

Recombinant Proteins—All recombinant proteins such as full-length P (amino acids 1–508), PNT (amino acids 1–291), PCT (amino acids 292–508), RPC (amino acids 316–382), and Px (amino acids 376–508) were expressed and purified to near homogeneity (Fig. 1). The authenticity of the purified proteins was confirmed by Western blot analysis using polyclonal antibody made against purified P protein expressed in E. coli (data not shown). As shown in Fig. 1, the full-length P and PNT migrate at positions corresponding to 80 and 52 kDa, respectively, which are much higher than their calculated masses (62 and 39 kDa, respectively). This anomalous mobility is attributed to the cluster of acidic residues at the N-terminal domain (18). Mass spectroscopic analysis of full-length P protein further confirmed its authenticity (data not shown).

**P Protein Exists as a Homo-oligomer in Solution**—The oligomerization state of bacterially expressed P protein was studied by SEC. As shown in Fig. 2, the majority of the P protein elutes at a position that corresponds to a molecular mass of more than 300 kDa. This result indicates that P forms a higher order multimer because the monomeric molecular mass is 62 kDa. The hydrodynamic radius of P protein was measured by DLS. The Rₐ of 7 nm for the P protein confirms the formation of an oligomer in solution. The chemical cross-linking experiment also suggested that the P protein exists as a multimer (data not shown).

**Coiled Coil Region on the C-terminal Domain Is Responsible for Oligomerization of P Protein into a Tetramer**—Recombinant proteins corresponding to the various parts of the P protein were subjected to SEC. Elution profiles are shown in Fig. 3. PNT (mass ~39 kDa) elutes at around 100 kDa, indicating that
it is either an oligomer or is a partially structured monomer. Earlier work had revealed that the C terminus is involved in P protein self-interaction (18). In the measles virus P protein, the equivalent domain (PNT) has been shown to be a partially structured monomer (34). Further, PCT (mass \( \geq 28 \text{ kDa} \)) eluted from the gel filtration column at a position of molecular mass \( 150 \text{ kDa} \). Taken together, these results suggest that the oligomerization domain lies at the C terminus of P (PCT).

The coiled coil region (RPC) and the rest of the C-terminal domain (Px) show molecular masses of 35 and 23 kDa, respectively, in SEC. This suggests that RPC (mass \( \leq 8.5 \text{ kDa} \)) is an oligomer, possibly a tetramer. The molecular size of Px (mass \( \leq 16 \text{ kDa} \)) is too small to be a dimer, and the increased molecular size of the monomer might result from its elongated shape or partially structured nature. The oligomeric state of RPC was further tested by DLS. The result showed an \( R_h \) of 2.8 nm (corresponding to \( \sim 34 \text{ kDa} \)) again confirming the oligomeric nature of RPC. To find out the exact stoichiometry of the RPC, chemical cross-linking of RPC was carried out. As shown in Fig. 4, in addition to monomers, cross-linked RPC was detected as dimers, trimers, and tetramers. With an increase in the duration of reaction and increase in cross-linker concentration, an increase in the number of tetramers was observed. Since cross-linked products higher than tetramer were not observed, we conclude that the most common form of RPC is a tetramer. The nature of interaction of such a tetramer was studied by CD spectral analysis in the presence and absence of trifluoroethanol (Fig. 5). These results indicated that RPC is rich in \( \alpha \)-helical content (\( \approx 90\% \)), and the ratio of ellipticities at 222/208 nm is greater than 1.0, indicative of the presence of interacting helices. Moreover, the ratio of ellipticities at 222/208 nm in 50% trifluoroethanol decreased to 0.918, characteristic of non-interacting \( \alpha \)-helices. Because trifluoroethanol has been shown to disrupt tertiary structure and quaternary structure and to promote secondary structure (35), this result suggests that RPC forms a coiled coil structure. Taken together, these results lead us to conclude that RPV P protein forms a tetramer through coiled coil interaction present in RPC.

**Fig. 3.** Elution profile of PCT (a), PNT (b), Px (c), and RPC (d) in gel filtration column. Purified proteins at 1 mg/ml were fractionated either on a Sephacryl S300 column (bed volume, 120 ml) or on a Sephadex G75 column (bed volume, 100 ml) as mentioned earlier. Protein content in each fraction (1 ml each) was monitored by \( A_{280} \) except RPC, which was monitored by estimating protein content of the fractions by Bradford assay. PCT, PNT, Px, and RPC eluted from the column at positions corresponding to 150, \( \leq 100, 23, \) and 35 kDa, respectively. The columns were calibrated as described in the text. The positions of different standard molecular mass markers are indicated on the top.

The biological function of the multimerization domain of P protein was assessed employing an *in vivo* replication/transcription system.
for RPV. As shown in Fig. 6a, the CAT protein level is significantly reduced when RPC is coexpressed with wild-type P protein compared with the control where the full-length P plasmid alone was used. Earlier studies in our laboratory have revealed that the coiled coil region does not interact with N protein (18) or L protein (36). These results clearly suggest that RPC forms a hetero-oligomer with wild-type P protein and thereby inhibits the function of the latter, because RPC lacks the other required functional domains. A similar result was observed when the PPRV P multimerization domain (amino acids 264–386) was used in the minigenome replication/transcription system in place of RPC (Fig. 6b). These results indicate that multimerization of P protein is essential for its function, and the multimerization domain is highly conserved between these two morbilliviruses. To further confirm this conservation of multimerization domain between the two viruses, the wild-type RPV P protein with the histidine tag at its N terminus was coexpressed with the untagged PPRV P multimerization domain in E. coli and purified by nickel affinity chromatography. As shown in Fig. 7, untagged PPRV P multimerization domain expressed alone could not be purified under similar conditions (data not shown). These results suggest that the multimerization domain of PPRV P can interact with RPV P signifying the conserved structure of this domain between the two viruses.

**Prediction of Secondary Structures and Coiled Coil Regions of RPV PMD**

The secondary structure prediction analysis using sequences that are closely related to the P protein showed that the multimerization domain is predominantly helical. In particular, the region from positions 316 to 355 is strongly predicted to be α-helical with a break of 4 residues around position 345. A few shorter segments of α-helix were also predicted in the beginning of the multimerization domain. Prediction of a long α-helical region is consistent with the fact that it could form a coiled coil structure.

The amino acid sequence of the multimerization domain was also subjected to the prediction of coiled coil regions. The regions from about 315 to 365 and from 310 to 375 are predicted as coiled coil regions. The result is shown in Fig. 8. It can be seen that the probability of a coiled coil structure for the region
of amino acids 340–360 is about 0.7 if the window size used in the program is 14. However, the estimated probability of coiled coil formation for other window sizes is suggested to be low (of the order of 0.2–0.4) probably because of the fact that these procedures do not consider the possibility of a four-stranded coiled coil. When the residues of RPV PMD in the heptad repeat positions (a–g) were analyzed, most of the nonpolar residues at a and d positions remained conserved. Although there were some drastic substitutions from nonpolar to polar residues at those positions, the interactions were maintained by compensatory changes, thereby maintaining the coiled coil structure. Such nonpolar to polar residue substitutions are also seen in many other coiled coil structures (37).

**Structural Features of RPV PMD**—The sequence identity between the multimerization domains of RPV P and SeV P is 14.3%. However if the similarity between the aligned residues in the two proteins are scored, the percent sequence similarity is 55.8%. This represents a low level of similarity, but appears convincing based on the compatibility of various residues in RPV P protein with the structural features in SeV P as well as the potential evolutionary relationship. Based on the comparative sequence analysis, it is very likely that the overall fold of the RPV PMD is similar to that of the crystal structure of SeV P. However, as the sequence identity (14.3%) is low, the details of the structures of these two proteins are likely to differ. The potential roles of various residues in the P protein in rendering stability to the coiled coil structure has also been investigated by generating a low resolution model using the crystal structure of SeV P as the basis.

The crystal structure of SeV PMD shows a tetrameric elongated structure. The N-terminal globular structure (approximately first 50 residues from each subunit) is followed by a parallel long coiled coil structure (21). The interaction between the protomers is present both in the small globular region as well as in the coiled coil region. The three-dimensional model for the amino acid sequence of RPV PMD protein generated using the COMPOSER suite of programs based on the alignment with SeV P is shown in Fig. 9.

A detailed examination of the model using interactive graphics suggests that the sequence of the multimerization domain of the RPV P protein could be comfortably accommodated in the fold of the SeV P multimerization domain despite a low sequence similarity between these proteins. Most of the apolar residues are buried in the structure, and polar residues are generally exposed. Many of the apparent drastic substitutions in the multimerization domain of RPV P protein compared with SeV PMD are accommodated by stabilizing interactions, which are different in nature compared with the observations in the crystal structure of SeV PMD.

Fig. 10 shows the amino acid sequences of SeV PMD and the
multimerization domain of the RPV P protein with the structural environment at every residue position of SeV PMD shown for both the tetrameric state (*crym*) and for a monomeric state (*crym*). The comparison of these two states show that the four-stranded coiled coil structural model of RPV PMD is largely stabilized by a series of interactions between the subunits involving short apolar side chains and a series of ionic pairs formed by oppositely charged amino acid side chains. This feature is also usually seen in the two- and three-stranded coiled coil structures (37). Many of the side chains that are involved in key interactions across the protomers of the tetramer model are shown in Fig. 9. The residues of SeV PMD that are exposed in the monomeric form, but get buried in the native tetramer form are given with the equivalent residue of the RPV PMD protein in the brackets (Fig. 10): Met-328 (Leu), Ser-332 (Thr), Ser-351 (Ser), Ala-355 (Gln), Ala-358 (Ile), Leu-359 (Glu), Cys-372 (Ile), Gly-373 (Gln), Leu-374 (Asp), Leu-376 (Lys), Ser-377 (Thr), Val-386 (Gln), Leu-393 (Leu), Ile-396 (Leu), Val-400 (Lys), Phe-403 (Ile), Tyr-407 (Lys), Gln-414 (Asn), and Leu-425 (Ser). This shows that in addition to the coiled coil region, the residues in the globular domain participate in intersubunit interactions. Some of the residues involved in intersubunit interactions are either conserved between the two proteins or substituted by another residue of similar chemical characteristics. However in a number of positions, the equivalent residues from the two proteins are significantly different.

Val-386 of SeV PMD is replaced by Gln in the RPV PMD. In the modeled structure Gln residues from the adjacent strands are oriented in such a way that a hydrogen-bonding network connecting the side chains is possible, contributing toward the stability of the four-stranded coiled coil structure. Another residue, Val-400, in the coiled coil region of SeV P is replaced by the lysine residue in the RPV P protein. Interestingly, in the model, the side chain of this lysine residue is hydrogen-bonded to the side chain of a glutamate residue from an adjacent strand, which is equivalent to Ser-402 of SeV P. Thus a cyclic network of salt bridges is predicted to stabilize the coiled coil structure. Similarly, the lysine residue of the RPV P protein that is equivalent to Tyr-407 of SeV PMD is hydrogen-bonded, through its side chain, with the side chain of an Asp in an adjacent strand. This Asp replaces Arg-404 of the crystal structure, and the net result of these two residue changes is yet another cyclic network of salt bridges linking adjacent strands. Thus, here is another example of apparently drastic amino acid substitutions (Tyr-407 → Lys and Arg-404 → Asp) resulting in the stabilizing interactions in the coiled coil structure. It is generally known that interactions between oppositely charged residues stabilize coiled coil structures (21). Leu-425 of the crystal structure occurs toward the end of the coiled coil structure and is replaced by Ser in the RPV P protein. In the model, the side chain -OH group of this Ser is hydrogen-bonded to the main chain carbonyl in the relative vicinity.

**DISCUSSION**

One of the long standing controversies is the exact oligomerization status of the P protein of negative-stranded RNA viruses. Studies on a number of viruses ranging from rhabdovirus to paramyxovirus report a different oligomeric status of P protein including dimer, trimer, and tetramer (9,11–13,17). By gel filtration, DLS, and chemical cross-linking analyses, we have shown that the RPV P protein exists as a tetramer in solution, and the tetramerization is through the coiled coil region of the C-terminal domain. Although earlier work on P proteins from SeV, NDV, and MuV suggested that P could be a trimer, recent structural analyses using various biophysical methods and crystallographic study reveal that SeV P is a tetramer (13, 21). Though VSV P protein is reported to be a trimer, it is also suggested to form a tetramer (9, 11). It might be noted that the study with rabies virus (a rhabdovirus) P also does not exclude the possibility of tetramer formation (12). All these results lead us to conclude that P proteins from this group of RNA viruses can exist as a tetramer.

P proteins exhibit differences in terms of their requirement of phosphorylation for multimerization. While the P protein of VSV and chandipura virus (ChP) (rhabdovirus) require phosphorylation to facilitate its oligomerization, P proteins from paramyxoviruses such as SeV, NDV, MuV, and MV have been shown to oligomerize independent of phosphorylation (7, 9, 17, 19). Moreover unlike VSV P and ChV P, rabies virus (another rhabdovirus) P protein can form an oligomer in the absence of any phosphorylation (12). The RPV P protein used in the present work is unphosphorylated as it was expressed in bacteria.
and could form a stable tetramer. These results suggest that multimerization of P protein is independent of phosphorylation except in the case of VSV and ChP. Notably, the study with VSV P also suggests the presence of a fraction of oligomeric population in the absence of any phosphorylation, and phosphorylation is only involved in the shift of equilibrium toward the multimer formation. Further, at high concentration, the unphosphorylated VSV P exists predominately as a multimer (16). Considering these results, it can be generalized that P proteins of mononegaloviruses exist as a multimer, possibly as a tetramer, whose oligomeric form is independent of any phosphorylation.

P proteins of mononegaloviruses also vary with respect to the role of phosphorylation on their biological functions. In SeV, phosphorylation of P has been shown to be dispensable for transcription and replication functions and a similar possibility is suggested for human parainfluenza virus 1 (hPIV1) also (1). Although phosphorylation of P has been reported to be essential for its activity in VSV and RSV, it has been shown that phosphorylation of HRSV P protein is not essential for its activity in VSV and RSV, more recently it has been suggested for human parainfluenza virus 1 (hPIV1) also (1). Although phosphorylation of P has been reported to be essential for its activity in VSV and RSV, there is no predicted coiled coil motif at its C terminus. This coiled coil motif might be responsible for multimerization involving possible tetramerization of P proteins among rhabdoviruses. Recently, with rabies virus P protein, it has been shown that the coated coil motif at the N-terminal domain is not involved in P protein multimerization and multimerization domain resides at the C-terminal domain, which also harbors the predicted coiled coil motif (12). However, in other rhabdoviruses like VSV, there is no predicted coiled coil motif at the C-terminal domain. This could mean that VSV P either oligomerizes through the N-terminal coiled coil motif or through the C-terminal domain which might harbor a coiled coil motif not detectable by prediction programs. Although further experiments are required to understand the multimerization of VSV P, our results on HPV P along with the results from other investigators discussed above suggest that there is a common mechanism for P protein function that acts as a multimer, possibly as a tetramer, and multimerization is independent of phosphorylation.

Acknowledgment—We thank the Department of Biotechnology, Government of India for infrastructural facilities.

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