Poliovirus 2C Protein Forms Homo-oligomeric Structures Required for ATPase Activity*

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Peter Adams‡, Eaazhaisi Kandiah‡, Grégory Effantin‡, Alasdair C. Steven§, and Ellie Ehrenfeld‡  
From the ‡NIAID and §NIAMS, National Institutes of Health, Bethesda, Maryland 20892-8011

The poliovirus protein 2C plays an essential role in viral RNA replication, although its precise biochemical activities or structural requirements have not been elucidated. The protein has several distinctive properties, including ATPase activity and membrane and RNA binding, that are conserved among orthologs of many positive-strand RNA viruses. Sequence alignments have placed these proteins in the SF3 helicase family, a subset of the AAA+ ATPase superfamily. A feature common to AAA+ proteins is the formation of oligomeric rings that are essential for their catalytic functions. Here we show that a recombinant protein, MBP-2C, in which maltose-binding protein was fused to 2C, formed soluble oligomers and that ATPase activity was restricted to oligomer-containing fractions from gel-filtration chromatography. The active fraction was visualized by negative-staining electron microscopy as ring-like particles composed of 5–8 protomers. This conclusion was confirmed by mass measurements obtained by scanning transmission electron microscopy. Mutation of amino acid residues in the 2C nucleotide-binding domain demonstrated that loss of the ability to bind or hydrolyze ATP did not affect oligomerization. Co-expression of active MBP-2C and inactive mutant proteins generated mixed oligomers that exhibited little ATPase activity, suggesting that incorporation of inactive subunits eliminates the function of the entire particle. Finally, deletion of the N-terminal 38 amino acids blocked oligomerization of the fusion protein and eliminated ATPase activity, despite retention of an unaltered nucleotide-binding domain.

Poliovirus is the prototype member of the Picornaviridae family. The 7.5-kb positive sense RNA genome encodes both capsid and noncapsid proteins that are necessary for virus replication. Translation of the viral genome into a single polyprotein yields both functionally distinct precursors and final products that are required for productive viral replication via an orchestrated series of co- and post-translational cleavage events catalyzed by viral proteinases. Replication of the viral RNA occurs in the cytoplasm, localized on the surfaces of newly formed membranous structures that develop after infection. Viral and host proteins involved in viral RNA replication form a poorly characterized, nuclease-resistant replication complex associated with the remodeled membrane structures. Numerous studies have demonstrated that viral protein 2C and its precursor 2BC play key roles in viral RNA replication, yet their actual biochemical functions in this complex reaction remain undefined (1–3). Protein 2C has been shown to interact with other elements of the viral replication apparatus, including 3AB (4), 3C proteinase (5), and the cloverleaf structure at the 5′-end of the viral genome (6). More recently it was shown that reticulon-3, a cellular protein involved in membrane trafficking and endoplasmic reticulum structure, binds polioviral as well as other picornaviral 2C proteins, and this plays an essential albeit undefined role in the process of virus replication (7).

Poliovirus 2C is tightly associated with intracellular membranes and can be cross-linked to actively replicating viral RNA isolated from infected cells (8). In addition, individual expression of 2C or 2BC in mammalian cells induces the formation of reorganized membrane structures from the endoplasmic reticulum, and thus these proteins have been implicated in the formation of virus-induced replication complexes (9, 10).

Poliovirus 2C is a 329-amino acid protein that is relatively highly conserved among members of the Picornaviridae family and is predicted to contain at least three domains (see Fig. 3) (11, 12). A centrally located nucleotide-binding domain (NBD) is the most highly conserved region of the protein. Flanking the NBD, at the N terminus of poliovirus 2C, is a region (amino acids 1–54) that contains a predicted amphipathic helix (amino acids 19–36) (13, 14) that specifies localization of 2C to the membrane (15). The C-terminal portion of the protein contains a small Cys-rich region that binds zinc (16), and a region that is thought to be involved in RNA binding and also may interact with membranes (12, 17). Biochemical studies of purified poliovirus 2C fusion proteins have demonstrated that the protein manifests an ATPase and a much weaker GTPase activity (18, 19). Mutations in NBD signature sequences result in impairment or abrogation of viral RNA replication when introduced into full-length or replicon viral RNAs (2, 20). The sensitivity of poliovirus RNA replication to millimolar concentrations of guanidine-HCl has been attributed to 2C: guanidine inhibits the ATPase activity of purified 2C protein in vitro, and viral mutants that are resistant or dependent for growth in the presence of guanidine harbor alterations in the NBD (21, 22). In other domains of the protein, mutagenesis by

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† To whom correspondence should be addressed: NIAID, NIH, 50 South Drive, Room 6120, Bethesda, MD 20892. Tel.: 301-594-1654; Fax: 301-435-6021; E-mail: eehrenfeld@niaid.nih.gov.

‡ The abbreviations used are: NBD, nucleotide-binding domain; MBP, maltose-binding protein; HRV, human rhinovirus; TEV, tobacco etch virus; EM, electron microscopy; STEM, scanning transmission electron microscopy; HCV, hepatitis C virus; GST, glutathione S-transferase.
insertion of sequences in the region C-terminal to the NBD causes temperature-dependent packaging defects (23), whereas gross changes to the upstream sequence in the region encompassing the predicted amphipathic helix also affect viral RNA replication (13). Taken together, these data imply that 2C is a multifunctional protein.

Comparative sequence alignments of the central NBD of picornaviral 2C proteins have grouped these proteins within the SF3 helicase family (24), although there is no evidence that poliovirus 2C protein has helicase activity or that such activity is necessary for virus replication (19, 25). The SF3 helicase family was originally identified in and appears to be limited to the genomes of DNA and small RNA viruses (26, 27). The NBDs of SF3 family members include the characteristic Walker A and B motifs along with a distinguishing C-motif (sensor) within a 100- to 120-amino acid region. The Walker A motif is specified by a GXXKG(T/S) signature, which interacts with the phosphates of ATP; the Walker B signature is defined by MDD, where the Asp (or Glu) residues interact with Mg$^{2+}$ or water and contribute to nucleotide hydrolysis activity. Motif C consists of an Asn residue preceded by a run of hydrophobic amino acids located C-terminal to the Walker B motif (27).

Although few biochemical or structural data are available for the picornaviral 2C proteins or other SF3 ATPases from RNA viruses, some SF3 helicase members encoded by DNA viruses have been quite well characterized (28, 29). These data indicate that DNA virus SF3 family proteins belong to the AAA+ superfamily, a functionally diverse group of proteins whose biological activities include protein folding, cytoskeletal regulation, and DNA replication (30). A common feature is the formation of higher order oligomers (predominantly hexamers and heptamers) that are essential for function. Indeed, structures of SV40 and papillomavirus E1 proteins resolved by x-ray crystallography revealed hexameric rings whose formation is dependent on the presence of ATP and magnesium (31, 32). These analyses showed that elements of two adjoining subunits are used to form the nucleotide binding site in the cleft between them. The nucleotide thus facilitates the formation and stability of a functional oligomer (33). Structure-based sequence alignment of these DNA SF3 helicases highlighted additional conserved residues that contribute to the nucleotide binding site in an adjacent protomer (29).

Poliovirus 2C differs significantly from SF3 DNA helicases, both in terms of overall size and properties such as membrane association. The low but significant homology among members of the SF3 family may indicate that there is commonality in biophysical properties such as oligomerization. The accumulated biochemical and genetic data for 2C oligomerization are contradictory. Yeast two-hybrid analysis of 2C proteins from polio (34) and related coxsackievirus (35) and teschovirus (36) failed to demonstrate a strong propensity for oligomerization of 2C, whereas biochemical studies (34) and a mammalian two-hybrid study (37) of poliovirus proteins suggested stable 2C-2C interactions may occur. Furthermore, genetic studies examining the mechanism of virus sensitivity or resistance to guanidine were interpreted as indicating that poliovirus 2C functioned as an oligomer (22). As part of our effort to understand the contribution(s) of 2C in the poliovirus replication cycle, we sought to determine the oligomerization status of the 2C protein and potential commonality with other related SF3 DNA helicases.

**EXPERIMENTAL PROCEDURES**

**Cloning and Mutagenesis of MBP-2C**—The gene encoding MBP was excised from the pMAL vector (New England Biolabs) with NdeI and XhoI. This fragment was inserted into pET28 expression plasmid that also had been digested with these restriction enzymes. The resulting expression plasmid encoded a hexahistidine at the N terminus of the MBP coding sequence to facilitate subsequent protein purification, and a 19-amino acid linker at the C terminus. The poliovirus (type 1, Mahoney strain) 2C gene, flanked by BglII and HindIII sites, was amplified from pTM7-PV2C (14) by PCR using the Phusion PCR kit (New England Biolabs). Sequences coding for the cleavage sites for either the human rhinovirus (HRV) 3C proteinase or the tobacco etch virus (TEV) proteinase at the N terminus of 2C were included in the 5′ oligonucleotide primer, preceding the 2C protein coding sequence. A truncated version of MBP-2C, where the 19-amino acid linker region between MBP and 2C was removed, was constructed to form pMAL28-2Calk and used for EM studies. The MBP-2C plasmid was digested with ScaI and EcoRI and re-ligated in the presence of a short section of duplex DNA with appropriate 5′- and 3′-ends. Deletion constructs that had 19 or 38 amino acids removed from the N terminus of 2C protein (Δ19 and Δ38, respectively) were constructed using pMAL28-2C as a PCR template, a 5′-oligonucleotide containing the appropriate deletion and a 3′-oligonucleotide containing a stop codon followed by HindIII site. Site-directed mutagenesis using a QuikChange kit (Stratagene) was employed to introduce mutations in the Walker A and B motifs of the 2C protein. Lys-135 was mutated to Ala in the Walker A motif, and Asp-178 was mutated to Ala in the Walker B motif in MBP-2C. Constructs with the HRV14 and HCV amphipathic helix substitutions were cloned from pPV2Cah-HRV14 and pPV2Cah-HCV (14), respectively, and inserted into the pMAL28 vector. Sequences of all individual clones were confirmed using a Big Dye terminator sequencing kit and automated sequencing with an ABI 3100 Gene Analyzer (Applied Biosystems).

**Protein Expression and Purification**—Protein expression was induced in *Escherichia coli* strain Rosetta (DE3) pLysS (Novagen). Fresh overnight cultures were inoculated into 1.5-liters of LB broth containing 50 mg/liter kanamycin and 34 mg/liter chloramphenicol and grown to OD$_{600}$ = 0.5. The flasks were then placed at 4 °C for 15 min before induction with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside at room temperature for 2 h. The pelleted bacteria were stored at −80 °C. The pellets were thawed with addition of Buffer A (20 mM HEPES, pH 7.5, 500 mM NaCl, 10 mM imidazole), which included 10 μg/ml DNase I, and Complete EDTA-free proteinase inhibitor tablets (Roche Applied Science). After three cycles of freeze/thaw, Triton X-100 was added to 1% and left for an additional 20 min on ice before centrifugation at 10,000 × g for 10 min. The clarified supernatant was applied to a column with a 4-ml bed volume of nickel resin (Qiagen) at 4 °C, collected, and reapplied. The column was washed with 100 column volumes of Buffer A/30 mM Mg$^{2+}$.
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imidazole. The MBP fusion protein was then eluted from the column using Buffer A/400 mM imidazole. The eluant was dialyzed against Buffer C (10 mM HEPES, pH 7.5, 200 mM NaCl, 5 mM dithiothreitol) and 2 mM EDTA, with two additional changes without EDTA, over 24 h to ensure the removal of bound nucleotides. The affinity-purified protein was loaded onto a Superose 6 3.2/30 gel-filtration column (GE Biotech) attached to a Biologic protein purification system (Bio-Rad) using Buffer C with a flow rate of 0.5 ml/min. The purity and identity of the sample were analyzed by SDS-PAGE and Western blotting with anti-2C monoclonal antibody (38).

Nucleotide Cross-linking—Cross-linking was carried out as previously described (39) with some modification: 10 pmol of purified protein was incubated in the presence of 1 μCi of [α-32P]ATP (400 Ci/mmol, Amersham Biosciences) in 10 mM HEPES, pH 6.8, 4 mM MgCl₂, and 5 mM dithiothreitol on ice for 15 min. The reactions were then transferred to a sheet of Parafilm on a block of ice and irradiated for 10 min at a distance of 8 cm from the lamp source of a Stratalinker (Stratagene). Following irradiation, individual samples were transferred to Eppendorf tubes, and an equal volume of SDS gel loading buffer was added. Samples were boiled for 5 min and resolved on a 12% PAGE-SDS gel. Gels were fixed, dried, and analyzed by autoradiography.

ATPase Assay—The reaction conditions used for ATPase assay were based on those described previously (16) with inorganic phosphate production detected with Biomol Green (40). Each 50-μl reaction contained 0.1 pmol/μl of protein, 0.2 mM ATP in 10 mM HEPES, pH 6.8, 5 mM dithiothreitol, and 4 mM MgCl₂. Samples were incubated at 37 °C for 30 min followed by the addition of 100 μl of Biomol Green (Biomol) and an additional 10 μl of 30% sodium acetate (41). Assays were carried out in a 96-well plate format, and absorbance was measured at 620 nm using a Molecular Dynamics plate reader.

Negative Staining for EM—Specimens were prepared using a conventional procedure. In brief, drops containing pMAL28-2CΔ2k protein (~100 μg/ml) in 50 mM HEPES, pH 7.0, 200 mM NaCl, 5 mM dithiothreitol, and 5 mM MgCl₂ were applied to glow-discharged carbon-coated copper grids and negatively stained with 1% uranyl acetate. The grids were examined with CM12 microscope (Phillips, Eindhoven, The Netherlands) at 120 kV. Low-dose micrographs were recorded on Kodak SO 163 films at a magnification of 45,000 X and digitized at a step size of 6.35 μm on a Nikon scanner, giving a pixel size of 1.41 Å.

Image Processing of Negative Stained Particles—Image analysis was done as described previously (42). Briefly, 1479 particles of MBP-2CΔ2k were picked manually, contrast transfer function-corrected by phase flipping and binned 2-fold, using BSOFT (43). The particles were centered and sorted into 20 class averages by reference-free classification as implemented in the EMAN Refine2D.py script (44). Depending on how well the images were resolved, 11 class averages were selected and used as seeds for further classification using SPIDER (45). The images were subjected to multireference alignment using 11 initial class averages, then sorted by correspondence analysis, using the first 10 (or so, in different runs) Eigen factors to classify the images by hierarchical ascendant clustering using Ward criteria. On examining the resulting dendogram, no clear cutoff was seen. Accordingly, several plausible cutoffs were chosen, generating different numbers of classes, and the resulting sets of class average images were calculated. Each set was examined visually, and the cutoff was chosen beyond which no additional features appeared in the class averages. These operations were repeated three times until a stable result was obtained. The final iteration yielded 13 class averages whose resolutions ranged from 28 to 5 Å as determined by Fourier ring correlation at 0.5 cutoff (46).

Scanning Transmission Electron Microscopy—The scanning transmission electron microscopy (STEM) images were recorded from grids prepared by the wet-film method (47) at Brookhaven National Laboratory. The digital images are of 512 × 512 pixels of 1 nm spacing between pixels. Tobacco mosaic virus was included as a mass standard. Masses were determined as described previously (48, 49), and the images were analyzed using the PCMass software (50).

RESULTS

Expression and Purification of MBP-2C—Previous efforts in this and other laboratories to express and purify poliovirus 2C protein for biochemical or structural studies have been thwarted by the protein’s inherent insolubility and aggregation properties. Fusion of 2C with MBP (42 kDa) at the 2C N terminus (19) generated good yields of soluble protein when expressed in a codon-enhanced strain of E. coli, Rosetta DE3 pLysS. This fusion protein, MBP-2C, manifested 2C-specific ATPase activity (see below), indicating that the 2C moiety retained at least some aspects of normal folding and conformation, and thus was considered acceptable for further characterization and analysis of catalytic, biochemical, and some structural properties. Purification of MBP-2C was facilitated by inclusion of a hexahistidine tag at the N terminus of MBP.

Fig. 1A, left panel, shows the SDS-PAGE profile of Coomassie Blue-stained proteins present in fractions from the total and soluble portions of the bacterial lysate. After purification on a nickel affinity column, a single major band migrating at the position of the predicted MBP-2C fusion protein (~82 kDa) was routinely obtained at a yield of ~2 mg/liter culture. The identity of the protein was confirmed by immunoblot with monoclonal anti-2C serum (Fig. 1A, right panel). Alternative fusions of 2C with glutathione S-transferase or thioredoxin resulted in less solubility, stability, yield, or purity (not shown).

Removal of the MBP moiety from the fusion protein following purification on the nickel column was achieved by inclusion of a cleavage site specific for the human rhinovirus 3C proteinase in the linker between MBP and 2C, followed by overnight incubation with the proteinase at 4 °C. Analysis of the 2C protein resulting from cleavage of the fusion was not pursued, however, because of low solubility, which in turn made further analyses problematic.

To examine the aggregation state of the MBP-2C fusion protein, affinity-purified material was dialyzed and applied to a Superose 6 gel-filtration column. The elution profile is shown in Fig. 1B. The major peak usually comprised 60–75% of the total material, eluting as a molecular mass of ~1000 kDa, and the remainder eluting as higher molecular mass complexes up to and greater than 2 mDa in the void volume. Only small
amounts of UV-absorbing material eluted at the position predicted for monomers or dimers (Fig. 1B). A small peak sometimes eluted with $M_r \sim 50$ kDa; this material did not react with anti-2C antiserum by immunoblot, and likely represents low level contaminants and/or degraded non-reactive 2C fusion fragments (Fig. 1D). It should be noted that the gel-filtration buffer did not contain magnesium and that extensive dialysis before chromatography should have minimized the presence of any nucleotide.

Individual fractions from the column eluate were assayed for ATPase activity. Fig. 1C shows that enzymatic activity was restricted to the major peak eluting $\sim 1000$ kDa (fractions 3 and 4 in Fig. 1B), with no evidence of activity associated with material eluting as lower molecular weight complexes, and only trace amounts of ATP hydrolysis occurring in fractions containing larger complexes of MBP-2C. This suggests that the latter material, eluting in the void volume or fractions 1 and 2 in Fig. 1B, likely represents misfolded aggregates of fusion protein, whereas the active form of the protein is an oligomer of MBP-2C subunits. These observations are consistent with data obtained for other characterized SF3 proteins, in which ATP binding and hydrolysis requires contributions from residues in adjacent subunits within an oligomeric structure (33, 51).

Although the relative amounts of the active peak versus the higher molecular weight aggregates varied in different preparations, the elution profile from a given preparation was unaffected by the addition of ATP/Mg$^{2+}$ (not shown).

The ATPase activity exhibited by the active peak from the gel filtration column was similar to that measured previously for a GST-2C fusion protein produced in E. coli (25). ATPase activity was completely inhibited by inclusion of 2 mM guanidine-HCl in the assay (not shown), confirming that the activity observed in these samples was attributable to the polioviral 2C moiety.

**Negative Staining of MBP-2C Oligomers**—To investigate the oligomeric status of the MBP-2C ATPase, we visualized the active peak fractions from gel-filtration columns by negative staining EM. Typical fields are shown in Fig. 2A. The micrographs depict somewhat heterogeneous populations of particles, with diameters mainly in the range of 150 to 200 Å. The relatively poor resolution of the size-exclusion gel within this mass range likely contributes to the heterogeneity of the population. Their substructure appears to consist of multiple lobes of density, $\sim 40$ Å across. A significant fraction of these molecules ($\sim 15\%$) appears to have a central disc, 50–70 Å in diameter, with a stain-penetrable lumen of 15–20 Å. Some examples are shown in Fig. 2D.

To assess the heterogeneity of these images in a more quantitative manner, we subjected them to image analysis, using classification by multivariate statistical analysis (52, 53) to identify sets of intrinsically similar images, and then averaging each of these sets to reduce the noise level. We experimented empirically with the number of sets (classes) to be distinguished and found this number not to be clearly defined, suggesting that there is residual heterogeneity (apart from the overlay of random noise) within each class. Nevertheless, certain trends emerged from the class average images that were essentially independent of class number ($>10$). Some class averages from an analysis of the data into 13 classes are shown in Fig. 2F.
The particles represented in the class average images vary in size diameter but typically have a peripheral fringe of globular lobes that vary somewhat in the regularity of their spacing, their density, the clarity with which they are resolved from their neighbors, and hence in their number. Some images also show similar non-peripheral features. At the center is a density of \( \sim 50-70 \) Å in diameter that varies somewhat in shape from class to class, and in some cases has a central stain accumulation. Classes 1 and 2 depict particles of 150–170 Å in diameter; the other classes are larger, at 170–200 Å. The smaller particles appear to have five or six peripheral lobes, and the larger ones, six to eight; however, we were unable to make an unambiguous count of the lobes in these images, for reasons given above. The lobes are 30–40 Å in diameter, matching the size of MBP (54). It is also not possible to know which of these populations contribute to the measured ATPase activity of the sample.

**Mass Measurements by STEM**—To further explore the subunit stoichiometries of MBP-2C, the masses of many individual molecules were measured by dark-field STEM imaging of unstained specimens (Fig. 2C). The distributions of mass measurements obtained for MBP-2C are shown in Fig. 2B. The distribution exhibits a broad, fairly symmetrical peak, centered around 500 kDa. A Gaussian curve fit of the data indicated an average molecular mass of 497 kDa (\( \sigma = 138 \) kDa). These values correspond to 6.1 ± 1.7 monomers for MBP-2C (monomer mass, 81.9 kDa). The relatively large standard deviation suggests that the stoichiometry is not uniquely defined (see below).

The EM analyses that we performed suggest that the MBP-2C oligomers are ring-like structures of variable stoichiometry, in which the subunits are connected by interactions between their ATPase domains, with the MBP moieties attached around the periphery (see below). In this scenario, the MBP appendage confers sufficient solubility to support assembly of the subunits into rings. Our inference that the rings are polymorphic is based on three observations: (i) the variation in size of the negatively stained molecules; (ii) the apparently variable numbers of lobes seen in the class averages; and (iii) the STEM data. Although the STEM distribution shows only a single peak, the peak is considerably broader than would be expected on theoretical grounds (50, 55) and in practice for proteins of similar mass observed in similar fashion (e.g. 550 ± 70 kDa for the p97 AAA+ ATPase (56)). Thus we interpret the single broad Gaussian peak observed for MBP-2C as a coalescence of multiple, unresolved, narrower Gaussian peaks and conclude that these populations include molecules with 5, 6, and 7 subunits, and possibly, some tetramers and octamers as well.

As to the proposed mode of oligomerization, MBP alone does not self-associate into rings but AAA+ ATPases, to which the picornaviral 2C ATPases show sequence relatedness, do. In many cases, they form cyclic hexamers (31, 57), but in other cases, rings whose numbers of subunits are not uniquely defined are formed (58); for instance, expression of ClpY (56) and ClpB (59) yields mixtures of hexamers and heptamers. When the SV40 T-antigen hexamer, for which a crystal structure is available, is limited to the same resolution as our class averages (30 Å), its top view projection has a donut-like appearance whose dimensions match quite well with those of the center densities in our class averages (cf. Fig. 2, E and F). Although the number of peripheral lobes can be counted with reasonable confidence for a few class averages (e.g. images #7 and #8 in Fig. 2F), in many cases they are not clearly defined. We attribute this property, which in most cases precludes determining stoichiometry by simply counting lobes, primarily to flexibility in the region of MBP fusion.
Oligomerization of MBP-2C Is Independent of ATP — Fig. 3 depicts a schematic diagram of the motifs and domains predicted to comprise the primary structure of poliovirus 2C protein. In the central portion of the protein, the sequences defining the Walker A, B, and C (WA, WB, and C, respectively) of the nucleotide binding domain are indicated. Conserved residues located in the WA (Lys-135) and WB (Asp-177) motifs were mutated to alanine, expressed as MBP fusions, and purified. The gel-filtration profile of the WA mutant is unchanged from that of wild-type MBP-2C (Fig. 4A). As expected, neither the WA nor WB mutant proteins exhibited ATPase activity (Table 1); however, when the impact of these mutations on ATP binding was measured directly by cross-linking experiments performed with the ~1000-kDa peak obtained by gel filtration (Fig. 4B), both wild-type and WB mutant displayed ATP binding, whereas the WA mutation almost completely abrogated detectable ATP binding. These data are consistent with the behavior of other AAA+ proteins, confirming the function of the WA motif in nucleotide binding and the WB motif in ATP hydrolysis (60). These results also reinforce the observations made above that oligomerization of MBP-2C is independent of ATP (in contrast to some other AAA+ proteins, such as ClpA, which require ATP to oligomerize (61)), because the gel-filtration elution profile was the same regardless of ATP binding.

N Terminus of 2C Determines Oligomerization of MBP-2C Fusion Proteins — The schematic diagram in Fig. 3 indicates a predicted amphipathic helix near the N terminus of the 2C sequences, which has been implicated in mediating the membrane binding property of 2C. Two MBP-2C fusion constructs were prepared in which N-terminal deletions were created that either spared (Δ19) or removed (Δ38) the amphipathic helix, and Fig. 5 shows their elution profiles. Removal of the N-terminal sequences, including the predicted amphipathic helix and the region preceding it, resulted in complete elimination of any oligomeric forms of MBP-Δ38 2C; in addition, no ATPase activity was detected (Table 1). The profile for the MBP-Δ19 2C mutant, which retained the amphipathic helix but deleted the preceding N-terminal sequences, shows that the presence of the amphipathic helix contributes to the formation of some higher order structures, although the elution pattern differed from that of the intact 2C fusion protein (compare Fig. 5 with Fig. 1B). We observed increased amounts of lower molecular weight forms and proportionally greater amounts of inactive high molecular weight aggregates. There was some variability in the relative amounts of different sized aggregates of MBP-2C among different preparations of this protein, perhaps indicating a greater sensitivity to misfolding and/or proteolysis. Simi-
lar to what was observed with the full-length MBP-2C, only a specific oligomeric form of Δ19 2C displayed any ATPase activity, and the specific activity was reduced (see Table 1). Thus, deletion of the N-terminal portion of 2C, including the predicted amphipathic helix, abrogated both oligomerization and ATPase activity; whereas the presence of the amphipathic helix, in the absence of the preceding N-terminal amino acid segment supported the formation of oligomeric complexes and some ATPase activity. These data suggested that the amphipathic helix may be a required determinant for oligomerization, whereas the adjacent N-terminal sequences may affect protein folding and/or stability.

Attempts to examine the role of the C terminus in oligomerization by deletion or substitution mutation analysis were thwarted due to either low levels of protein expression or increased levels of protein degradation. Although there was some evidence for oligomerization of MBP-2C proteins with C-terminal deletions, the ATPase activity was very low (data not shown).

Role of the Amphipathic Helix in Assembly of Active 2C Oligomer—The experiments shown in Fig. 5 and discussed above pointed to an important role for the N terminus, which includes an amphipathic helix, in the formation of active oligomeric MBP-2C protein. Our laboratory has described previously the construction of 2C chimeric proteins in which the orthologous amphipathic helices from either HRV 2C or hepatitis C virus (HCV) protein NS5A were substituted for the poliovirus 2C amphipathic helix (14). Viral genomes encoding the HRV 2C chimera were viable, albeit slowly replicating, whereas those encoding the HCV 2C chimera did not replicate. These previous results showing restriction on helix exchangeability indicated that the amphipathic helix, although representing a relatively independent module that had been conserved in at least two different RNA virus families to confer similar properties of membrane anchoring for viral replication proteins (14), nevertheless also contributed to other functions requiring interaction with other viral protein sequences.

We constructed MBP fusion proteins encoding each of these two chimeric 2C proteins, MBP-2Cah-HRV14 and MBP-2Cah-HCV, that were purified and analyzed by gel filtration. The elution profiles are shown in Fig. 6. Both profiles were similar to that of wild-type polio MBP-2C (Fig. 1B), although immunoblots of fractions eluting from the gel-filtration column detected increased levels of 2C-containing material in the eluate corresponding to monomeric or dimeric MBP fusions with the chimeric proteins (data not shown), which did not occur in the wild-type 2C preparations (Fig. 1B). Fractions corresponding to the ~1000-kDa peak showed levels of ATPase activity comparable to the wild-type fusion proteins (Table 1). Thus, inclusion of the amphipathic helical region is essential for both membrane binding and oligomerization of 2C, and its specific amino acid sequence had no effect on the nucleotide binding or ATPase activity of the oligomeric protein. The possibility that insertion of a “spacer sequence” that lacked amphipathic helical properties might still rescue the oligomerization and ATPase activities in the chimeric constructs was not addressed.

Co-expression of Wild-type and Mutant MBP-2C Protomers—If assembly of the 2C moieties into an oligomeric structure is required for ATPase activity to be manifested, then co-expression of wild-type MBP-2C and MBP fusion proteins harboring mutations that prevent ATP binding or hydrolysis under conditions where oligomers containing a mixed population of subunits could form might be predicted to exert non-additive or dominant negative effects on activity. We co-expressed wild-type polio MBP-2C together with WA or WB mutant MBP-2C proteins, purified the fusion proteins from clarified bacterial lysates by nickel affinity column chromatography, and determined the levels of ATPase activity of each preparation. To allow us to determine the relative amounts of wild-type and mutant fusion protein expressed in the co-expression cultures,
we utilized a wild-type construct containing an HRV 3C proteinase cleavage site inserted in between the MBP and 2C moieties. The mutant WA and WB mutant fusions contained TEV proteinase cleavage sites that were not cleavable with HRV proteinase. As predicted, the oligomeric proteins isolated from bacteria co-expressing both wild-type and either WA or WB mutant fusions exhibited significantly less activity than expected from the proportion of wild-type protein in the sample. Indeed, the presence of even low amounts of the WA mutant exhibited a strong dominant negative effect on the activity exhibited by the wild-type protein, eliminating virtually all detectable hydrolysis of ATP by the hybrid protein sample (Fig. 7C).

**DISCUSSION**

**MBP 2C Analysis**—We have undertaken biophysical characterization of 2C to establish if there are commonalities with the related and more fully characterized members of the SF3 DNA helicase family. The relatively small size of the 2C proteins compared with other SF3 DNA helicases, along with the presence of distinct accessory domains such as membrane binding determinants, complicate the assignment of functional equivalency with SF3 DNA helicase proteins.

During poliovirus infection, 2C protein is associated with membranous viral replication complexes (8). When expressed in mammalian or bacterial cells, 2C associates tightly with available membrane structures, which impedes isolation of soluble protein and restricts approaches convenient for biochemical analysis. Previous attempts to recover soluble and catalytically active poliovirus 2C protein succeeded only when it was fused to additional polypeptide such as GST or MBP (19, 25). In our studies, we utilized 2C attached to the carboxyl end of MBP to recover protein in a form capable of catalytic ATPase activity. MBP fusions have been used previously for biochemical and structural analyses of a number of proteins that were otherwise intractable for biochemical analysis (e.g. Ref. 62 and refs within). Here we used a number of different approaches to show that poliovirus MBP-2C fusions display functional ATPase activity only in an oligomeric structure. Although gel filtration showed that MBP-2C protein consisted of a heterogeneous population of different sizes, the catalytically active fraction eluted as an oligomer with an estimated mass of ~1000 kDa, whereas STEM analysis suggested a value closer to ~500 kDa. The discrepancy between these values may be due to aberrant retention of the hydrophobic protein on the gel filtration matrix. Visualization of the protein complexes by EM after negative staining revealed rings of 5–8 monomers, consistent with the molecular mass range estimated by STEM analysis. Modi-
Oligomeric Poliovirus 2C Protein

The 2C portion of the fusion protein is responsible for its oligomerization and catalytic function, attributing these properties to the 2C rather than the MBP moiety. In addition, GST-2C fusion proteins also formed multimERIC structures with similar specific ATPase activities. The latter were relatively unstable and were not characterized further.

Relationship of Nucleotide Binding and Oligomerization—Structural and biochemical data from individual SF3 DNA helicases show that they share a core architecture, despite relatively limited sequence homology. The SF3 helicases are part of the larger grouping of AAA++ proteins, in which oligomerization is a characteristic functional feature. In the assembled particles, the NBD is located between individual subunits with both protomers contributing to the formation of a functional binding site. In the well characterized SV40 (31) and papillomavirus E1 (32) DNA helicases, the assembly of a functional hexamer is dependent upon ATP binding. We constructed MBP-2C proteins with mutations in the WA and WB motifs to determine the dependence of oligomerization on ATP binding for the polioviral 2C protein. By analogy with the properties of other AAA++ proteins, we predicted that mutation in the WA motif would prevent nucleotide binding, whereas mutation of the WB motif would retain ATP binding but impair ATP hydrolysis. These predictions were confirmed by ATP cross-linking and ATPase activity assays of MBP-2C WA mutant and the MBP-2C WB mutant remained predominantly oligomeric, despite their differing abilities to bind ATP. The gel-filtration profile of MBP-2C WA mutant was not significantly different from the wild-type protein. Although this may be unusual, there are other examples of AAA++ proteins that form oligomers in the absence of nucleotide and magnesium. For example, the RF100 phage helicase remains predominantly hexameric in the absence of ATP, apparently due to an unusual arrangement and interlinking of the C terminus with the adjacent protomer (63). Additional evidence to support the correlation between oligomerization and ATPase activity of 2C was obtained by co-expressing wild-type and mutant forms of the fusion proteins, which produced a dominant negative effect on enzymatic activity. A similar result was obtained with AAV Rep 40, which is a member of the SF3 DNA helicase family (64).

Oligomerization Is Mediated by the N Terminus of 2C—Cystal structures of SF3 DNA helicases provide an insight into the role that the region N-terminal to the NBD has in the formation of a functional oligomers of 2C. Two MBP fusion constructs with N-terminal deletions that either retained the amphipathic helix (Δ19) or deleted it (Δ38), demonstrated the importance of the N terminus in the formation of functional oligomers of MBP-2C. Gel filtration showed that removal of the first 38 amino acids resulted in complete loss of any oligomeric forms of the protein, as well as any detectable ATPase activity. Removal of only the first 19 amino acids also had an effect on ATPase activity, accompanied by more subtle changes in the gel-filtration profile that revealed increased monomeric/dimeric forms along with higher molecular weight aggregates.

These data are consistent with previous studies showing that removal of the first 42 amino acids from the N terminus of 2C resulted in loss of ATPase activity (17), which can now be explained by loss of the ability to form a functional oligomer.

The N-terminal region of poliovirus 2C contains a predicted amphipathic helix (residues 19–36) (13, 14) that mediates membrane association (15). An orthologous amphipathic helix was identified in the N-terminal sequence of the NS5A protein of hepatitis C virus, a member of the Flaviviridae family of positive-strand RNA viruses (65). This region from the latter viral protein has been demonstrated to form an amphipathic helix that lies across the face of the membrane. The helix is positioned within the lipid bilayer such that charged residues are arranged along a face and interact with the lipid headgroups (66). We have previously shown that substitution of the sequences specifying the predicted amphipathic helix in poliovirus 2C protein with the amphipathic helices derived from the equivalent regions or HCV or other members of the Picornaviridae family affected overall virus replication (14). When the MBP-2C proteins containing the HCV NS5A or HRV 2C amphipathic helices were examined for protein oligomerization and ATPase activity, we found that both of these properties were unaffected by substitution of the amphipathic helix. Therefore, membrane binding, oligomerization, and catalysis of ATP hydrolysis were dependent on the presence of the amphipathic helical region, but are not sufficient for polioviral protein 2C to carry out its multiple roles in viral RNA replication.

Origins of Polymorphism in MBP 2C—As noted above, there are precedents for polymorphism in some AAA++ proteins when overexpressed in recombinant systems; e.g. ClpY and ClpB have been observed to yield mixtures of hexamers and pentamers under similar conditions. In this respect, recombinant MBP-2C is even laxer in specifying its stoichiometry, because our data indicate that it may form some pentamers and octamers as well. The variability may be compared with that exhibited by portal protein from tailed bacteriophages and herpesviruses. These are also ring-like oligomers that serve as a channel for the passage of duplex DNA into capsids. It appears that portals incorporated into assembling virions in vivo are invariably 12-mers. In contrast, recombinant expression can produce mixtures of 12-mers and 13-mers for the T7 protein (67); mainly or all 13-mers for SPP1 (68); mixtures of 11-mers and 12-mers for P22 (69); or rings with 11 to 14 subunits in the case of HSV-1 (70). It may be, therefore, that fusing the MBP to 2C, while rendering the protein soluble and functionally competent as an ATPase, made the protein laxer in specification of its stoichiometry. The oligomeric character of 2C under conditions of native expression remains to be seen. Although numerous biochemical and genetic studies demonstrate the importance of poliovirus 2C protein in the process of viral RNA replication, it is not yet known what functions these oligomeric properties of 2C protein contribute to the reactions occurring in the viral RNA replication complexes.

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3 P. Adams, E. Kandiah, G. Effantin, A. C. Steven, and E. Ehrenfeld, unpublished data.
