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Effect of Osmolytes and Chaperone-like Action of P-protein on Folding of Nucleocapsid Protein of Chandipura Virus*

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Amitabha Majumder‡§, Soumen Basak‡§, Tamal Raha‡, Santanu Pal Chowdhury‡, Dhrubajyoti Chattopadhyay‡¶, and Siddhartha Roy‖**

From the ‡Department of Biochemistry and the Dr. B. C. Guha Centre for Genetic Engineering and Biotechnology, University College of Science, University of Calcutta, 35 Ballygunge Circular Road, Calcutta 700 019, India and the ‖Department of Biophysics, Bose Institute, P 1/12 CIT. Scheme VII M, Calcutta 700 054, India

Amino acid sequences of nucleocapsid proteins are mostly conserved among different rhabdoviruses. The protein plays a common functional role in different RNA viruses by enwrapping the viral genomic RNA in an RNase-resistant form. Upon expression of the nucleocapsid protein alone in COS cells and in bacteria, it forms large insoluble aggregates. In this work, we have reported for the first time the full-length cloning of the N gene of Chandipura virus and its expression in *Escherichia coli* in a soluble monomeric form and purification using nonionic detergents. The biological activity of the soluble recombinant protein has been tested, and it was found to possess efficient RNA-binding ability. The state of aggregation of the recombinant protein was monitored using light scattering. In the absence of nonionic detergents, it formed large aggregates. Aggregation was significantly reduced in the presence of osmolytes such as D-sorbitol. Aggregate formation was suppressed in the presence of another viral product, phosphoprotein P, in a chaperone-like manner. Both the osmolyte and phosphoprotein P also suppressed aggregation to a great extent during refolding from a guanidine hydrochloride-denatured form. The function of the phosphoprotein and osmolyte appears to be synergistic to keep the N-protein in a soluble biologically competent form in virus-infected cells.

The (−)-strand RNA viruses (rhabdovirus, influenza, rabies, measles, and Ebola) consist of important human pathogens, including Chandipura virus (CHPV),[1] a member of the Rhabdoviridae family (1). The structure, function, and genetic makeup of this negative sense RNA virus resemble those of vesicular stomatitis virus (VSV). Still, it can be distinguished from two other members of the rhabdovirus family, VSV New Jersey serotype and VSV Indiana serotype, not only in its host species origin and serology (2), but also in nucleotide sequences of genes and amino acid sequences of proteins (3). Upon infection by members of the rhabdovirus family, five major viral proteins, N (nucleocapsid), P (phosphoprotein), L (large), M (matrix), and G (glycoprotein), are synthesized. The N-protein encapsidates genomic RNA in a precise structure that can be compared with histone-mediated enwrapping of a DNA molecule into a nucleosome structure. Only this encapsidated form of the genome can be recognized by viral polymerase as its template during both transcription and replication (4). Nucleocapsid proteins not only protect the viral genome from RNase action, but are also thought to play some vital regulatory roles in the transition from transcription to replication in the viral life cycle, referred to as the transcription-replication switch.

Previous studies have indicated that the N-protein has a common tendency to form large aggregates that are biologically inactive. In VSV, it was observed that interaction of the N-protein with the P-protein keeps the N-protein in a soluble form *in vivo* that is capable of enwrapping the *de novo* synthesized genomic RNA. N-protein/P-protein interaction also may confer specificity for RNA-binding activity (5) of the N-protein where it channels the N-protein pool to viral RNA sequences only. This regulatory importance of N-protein/P-protein interaction makes it an important biological event in the viral life cycle. In influenza virus, the nucleoprotein was found to interact with viral RNA polymerase, directly modulating its activity (6). In VSV, it has been proposed that the P-protein in its phosphorylated multimeric state forms a complex with the L-protein to produce functional transcriptase, whereas in its unphosphorylated state, it complexes with the L-protein to form replicase. The N-protein has been suggested to be an integral member of the replicase complex (7). The multifunctional nature of the N-protein and its interaction with different targets make this viral product an attractive model for a detailed structure-function analysis in the CHPV system so that its precise role in different stages of the viral life cycle can be elucidated.

One of the important aspects of N-protein function is the maintenance of its active and soluble form. As mentioned before, in its free form, the protein has a tendency to form aggregates *in vitro* and perhaps *in vivo* (8). How the cellular environment maintains a soluble and active pool of the N-protein is crucial to understanding the roles played by various cellular factors in the viral life cycle. Clearly, the P-protein increases the solubility of the N-protein *in vivo* through formation of complexes. However, the structural nature of the N-protein in these complexes is not known. Thus, the possibility is raised that the P-protein may be interacting with a disordered N-

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§ Both authors contributed equally to this work.
¶ To whom correspondence may be addressed. Tel.: 91-33-334-9382; Fax: 91-33-476-4419; E-mail: djc@netkracker.com.
** To whom correspondence may be addressed: Tel.: 91-33-412-1261; Fax: 91-33-334-3886; E-mail: sidroy@vsnl.com.

[1] The abbreviations used are: CHPV, Chandipura virus; VSV, vesicular stomatitis virus; I-EDANS, 5-(2-iodoacetylaminoethyl)naphthalene-1-sulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyranoside; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DLS, dynamic light scattering; dansyl, 5,5′-dithiobis(2-nitrobenzoic acid).

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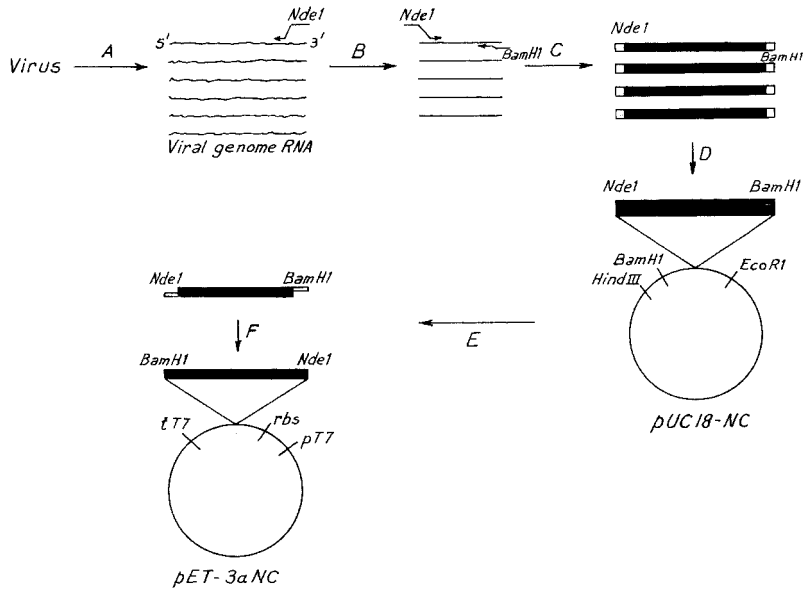

FIG. 1. **Cloning of the N gene.** *Step A*, isolation of viral RNA; *step B*, first-strand cDNA synthesis with the DJC1 oligonucleotide using Superscript II reverse transcriptase; *step C*, PCR of cDNA with primers DJC1 and DJC2, followed by blunt-ending of the PCR product with Klenow polymerase in the absence of dNTPs; *step D*, cloning of the reverse transcription-PCR product into *Sma*I-digested pUC18 vector; *step E*, restriction digestion of pUC18-NC with *Nde*I and *Bam*HI and isolation of the N gene-containing fragment; *step F*, subcloning of the N gene into the pET-3a vector digested with *Nde*I and *Bam*HI under control of the T7 promoter (*pT7*). *tT7*, T7 terminator; *rbs*, ribosome-binding site. The *solid bar* represents the N gene open reading frame.

protein in a chaperone-like fashion. Protein folding studies *in vivo* have elucidated the crucial roles of osmolytes (9) and chaperone systems in suppressing aggregation and shifting the distribution toward the folded state, thereby enhancing the activity and solubility of the proteins. A central point in this study is to understand the roles of the P-protein and intracellular osmolytes in maintaining and enhancing the solubility of the N-protein, thereby ensuring a steady supply for encapsidation of progeny viruses.

EXPERIMENTAL PROCEDURES

Materials—*Pfu* DNA polymerase and T4 DNA ligase were from New England Biolabs (Beverly, MA). Tripure reagent™ and Superscript II reverse transcriptase were from Life Technologies, Inc. Klenow polymerase, SP6 RNA polymerase, DNase, 4-nitro blue tetrazolium chloride, and 5-bromo-4-chloro-3-indolyl phosphate were from Roche Molecular Biochemicals. The gel extraction kit was from QIAGEN Inc. The Mono-Q column was from Amersham Pharmacia Biotech (Uppsala, Sweden). Alkaline phosphatase-conjugated goat anti-mouse IgG was from Sigma. I-EDANS was from Molecular Probes, Inc. (Eugene, OR). Prestained molecular mass markers and Bio-Beads SM-2 adsorbent were from Bio-Rad. Other common reagents were of analytical grade.

Construction of the Full-length Clone of the N Gene of CHPV by Isolation of RNA—The viral genomic RNA was isolated from CHPV (strain 1653514) using Tripure reagent™ according to the manufacturer's protocol. Isolated RNA was estimated qualitatively and quantitatively by running 1% MOPS gel and by spectrophotometric analysis at 260 and 280 nm, respectively.

Reverse Transcription-PCR to Amplify the N Gene of CHPV—Two terminal primers (DJC1 and DJC2) were designed to clone the N gene by reverse transcription-PCR. Primer DJC1 (5′-TTTATA CATATG AGTTCTCAAGTA-3′) included a hexameric anchor followed by an *Nde*I restriction enzyme site. The rest of the sequence corresponds to the 5′ terminus of the N gene. In primer DJC2 (5′-TTTATA GGATCC TCATGCAAAGAG-3′), the hexameric anchor was followed by a *Bam*HI restriction enzyme site, and the rest of the sequence was complementary to the 3′-end of the N gene (3). First-strand cDNA synthesis was carried out with primer DJC1 and viral genomic RNA as template using Superscript II reverse transcriptase, and PCR was done with *Pfu* polymerase according to the instructions given by the manufacturer. The PCR product was run on a 1% agarose gel in 0.5× Tris borate/EDTA (Fig. 1).

Cloning of the N Gene in the pUC18 Vector—The PCR product purified from the 1% agarose gel using QIAGEN gel extraction resin was blunt-ended with Klenow polymerase and cloned into *Sma*I-digested pUC18 vector using T4 DNA ligase. The ligation mixture was transformed into *Escherichia coli* XL1-Blue, and positive clones were confirmed by restriction digestion, followed by direct sequencing of the recombinant DNA (Fig. 1).

Subcloning of the N Gene in the pET-3a Vector—The full-length N gene of CHPV was digested in the pUC18-NC clone with *Nde*I and *Bam*HI restriction enzymes, and the released DNA fragment was subcloned into *Nde*I/*Bam*HI-cut pET-3a vector under the control of the T7 promoter. The recombinant DNA was digested with different restriction enzymes for further confirmation (Fig. 1).

Expression of the N gene in E. coli—Competent *E. coli* BL21(DE3) cells were transformed with pET-3a-NC plasmid DNA, and transformed cells were inoculated in 100 ml of Luria broth supplemented with 20 mM glucose containing 100 mg/ml ampicillin and incubated at 37 °C under shaking conditions until A_{600} = 0.3. Cells were then induced with 500 μM IPTG for 4 h at 37 °C (10). After harvesting, the cell pellet was suspended in 2 ml of buffer A containing 50 mM Tris-HCl, 1 mM EDTA, and 0.1% Triton X-100. For lysis, lysozyme was added at a concentration of 250 μg/ml to buffer A, and the suspension was kept on ice for 1 h. The cells were then sonicated to reduce the viscosity due to chromosomal DNA, and lysates were clarified by spinning at 13,000 rpm for 30 min at 4 °C. The soluble supernatant was separated from the inclusion body pellet. The inclusion body pellet obtained after centrifugation was denatured and taken into 2 ml of a solution of 8 M urea in buffer A. The soluble and urea fractions were analyzed by 10% SDS-polyacrylamide gel electrophoresis, followed by Coomassie Blue staining. Different parameters such as IPTG concentration, temperature, and duration of induction were varied to maximize expression of the N-protein in soluble form.

Purification of the Bacterially Expressed N-protein—The N-protein expressed in soluble form was purified from bacterial lysate through a Mono-Q anion-exchange fast protein liquid chromatography column. The column was pre-equilibrated with buffer A containing 200 mM NaCl, and proteins were eluted with a gradient of NaCl from 200 to 700 mM in a total volume of 25 ml of buffer A. The flow rate and fraction size were 0.25 ml/min and 1 ml, respectively. Fractions were analyzed on 10% discontinuous SDS-polyacrylamide gel (acrylamide/bisacrylamide ratio of 30:0.8), followed by Coomassie Blue staining to test the homogeneity of the purified protein. The purified N-protein was stored in buffer A containing 200 mM NaCl and 10% glycerol at −20 °C

Western Blot Analysis—Proteins were subjected to 10% SDS-polyacrylamide gel electrophoresis, and Western blotting was performed with mouse polyclonal anti-CHPV antibody as the primary antibody and alkaline phosphatase-conjugated goat anti-mouse IgG as the secondary antibody. This was followed by color reaction with 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate.

Synthesis of ^{32}P-Labeled Leader RNA—A 49-nucleotide leader RNA gene of CHVP was cloned under the control of the SP6 promoter in the pGEM-4Z vector. DNA template was linearized with the *Hin*dIII restriction enzyme. Radiolabeled positive sense leader RNA was synthesized *in vitro* in a 40-ml reaction with [α-^{32}P]UTP and SP6 RNA polymerase essentially as described by the manufacturer. DNA template was removed by RQ1 DNase treatment, and the leader RNA was extracted with phenol/chloroform and precipitated twice with ethanol. The product was analyzed on 10% polyacrylamide gel (29:1) containing 8 M urea in 1× TAE (40 mM Tris-acetate, 2 mM EDTA, pH 8.0).

In Vitro Encapsidation of Leader RNA—To study the encapsidation

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reaction, ^{32}P-labeled leader RNA (200 ng) was incubated with increasing amounts of N-protein (0.3–4.5 μM). The binding reaction was carried out in a total volume of 15 ml in 10 mM Tris-HCl containing 100 mM NaCl, 40 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 5% glycerol at 37 °C for 30 min. In the presence and absence of UV cross-linking (Appligene UV crosslinker), the RNA-protein complex was treated with RNase A at a final concentration of 60 mg/ml for 15 min. The reaction was stopped by the addition of 10× loading dye containing 30% Ficoll, 1 mM EDTA, 0.25% bromphenol blue, and 0.25% xylene cyanol. The reaction mixture was run on a 6% nondenaturing polyacrylamide gel containing 5% glycerol at 4 °C in TAE (40 mM Tris-acetate, 2 mM EDTA, pH 8.0) (acrylamide/bisacrylamide of 30:0.8). The gel was dried and exposed to x-ray film at −70 °C.

Expression and Purification of the Recombinant P-protein from Bacteria—The P-protein of CHPV was expressed in bacteria, and only the soluble fraction was purified as described earlier (11).

Determination of Sulfhydryl Content by DTNB Titration—To determine the number of reactive sulfhydryl groups by DTNB titration, 10 μM CHPV N-protein was incubated with 0.5 mM DTNB in 50 mM Tris-HCl (pH 8) at 25 °C. The reaction was monitored at 412 nm in a Hitachi UV-2000 spectrophotometer after an appropriate base-line correction. A molar extinction coefficient of 1.36×10^4 M^{-1} cm^{-1} was used to calculate the number of reactive sulfhydryl groups.

Labeling of the CHPV N-protein with I-EDANS—The purified CHPV N-protein (10 μM) was reacted with 5 mM I-EDANS at 37 °C for 2 h. After incubation, the protein was dialyzed against 50 mM Tris-HCl (pH 8) containing 150 mM NaCl to remove the unreacted I-EDANS. The absorbance at 337 nm was measured to check the incorporation of I-EDANS into the protein with the buffer from the previous dialysis being used for the base line.

Circular Dichroism Spectroscopy—Circular dichroism spectra were measured in a Jasco J-700 spectropolarimeter. Measurements were carried out in 50 mM Tris-HCl (pH 8) containing 150 mM NaCl at ambient temperature. An average of four scans was taken. A time constant of 2 s and a scan speed of 50 nm/min were used for spectral scanning. The protein concentration was 10 μM. Proper base-line corrections were made.

Measurement of Steady-state Fluorescence Anisotropy—Anisotropy experiments were performed using a Hitachi polarization accessory. The fluorescence intensity components I_{vv}, I_{vh}, I_{hv}, and I_{hh} (where the subscripts refer to the horizontal (h) and vertical (v) positioning of the excitation and emission polarizers, respectively) were used to calculate the steady-state fluorescence anisotropy (A) according to the following equation: $A = (I_{vv} - GI_{vh})/(I_{vv} + 2GI_{vh})$, where G is the grating factor that corrects for the wavelength-dependent distortions of the polarizing system.

Fluorescence Spectroscopy—Steady-state fluorescence spectra were recorded in a Hitachi F-3010 spectrofluorometer with spectrum addition and subtraction facility. The fluorescence experiments were carried out at 37 °C, and the temperature was maintained by a circulating water bath attached to the spectrofluorometer. The excitation and emission band passes were maintained at 5 nm, and all readings were taken in a cuvette with a 1-cm path length.

Static Light Scattering—Light scattering experiments were done to check the state of aggregation of the soluble CHPV N-protein and to monitor the roles of osmolytes and the CHPV P-protein in this aggregation process. The CHPV N-protein at a high concentration was first treated with Bio-Beads SM-2 adsorbent to remove Triton X-100, filtered three times through a Millipore filter, and added to a Millipore filtered buffer under suitable dust-free conditions to a final protein concentration of 50 μM. All additions were made in the cuvette, and light scattering was measured with excitation and emission wavelengths set at 340 nm. To determine the effect of osmolytes, the same experiment was carried out, but a 250 mM concentration of the osmolyte D-sorbitol was added during detergent removal from a high concentration of CHPV N-protein. To determine the effect of the CHPV P-protein on aggregation, the concentrated stock of detergent-removed CHPV N-protein was denatured with 8 M urea. The denatured protein was diluted in a native buffer in the fluorometer cuvette, and scattering intensities were monitored in the presence of varying concentrations of CHPV P-protein and osmolytes.

Dynamic Light Scattering—Dynamic light scattering experiments were performed using an Otsuka Electronics DLS700 instrument. For DLS experiments, the concentration of CHPV N-protein was kept at 1 mg/ml, and the concentration of D-sorbitol was kept at 250 mM. In this experiment, the sample was illuminated with a 638.8 helium-neon solid-state laser, and the intensity of light scattered at an angle of 90° was measured. An auto-correlation function was used to determine the translational diffusion coefficient (D_T) of the sample particles in the solution by measuring the fluctuations in the intensity of the scattered light. The hydrodynamic radius (R_H) of the sample particles was derived from D_T using Stokes-Einstein's equation: $D_T = k_B T/6\pi\eta R_H$, where k_B is the Boltzmann constant, T is the absolute temperature in degrees Kelvin, and η is the solvent viscosity.

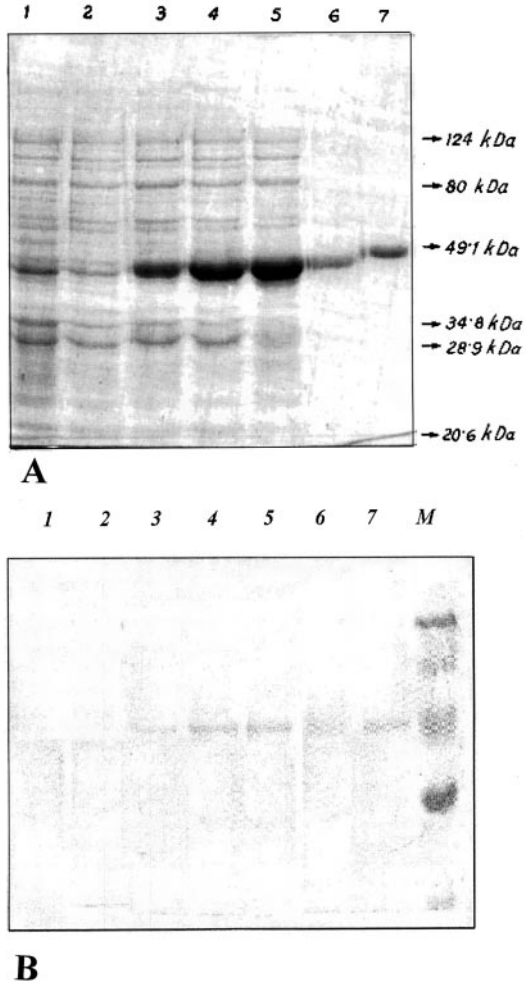

FIG. 2. **Expression and purification of the recombinant N-protein in *E. coli*.** *Lanes 1* and *2*, total protein from cells transformed with pET-3a and uninduced or induced with IPTG, respectively; *lanes 3* and *4*, BL21(DE3) cells (pET-3a-NC) uninduced and induced with IPTG, respectively; *lane 5*, soluble supernatant fraction from IPTG-induced cells; *lane 6*, inclusion bodies extracted with 8 M urea from IPTG-induced cells; *lane 7*, N-protein purified through a Mono-Q column (fast protein liquid chromatography). *A*, Coomassie Blue-stained 10% SDS-polyacrylamide gel showing the expression and different stages of purification of the recombinant N-protein. Protein molecular mass markers are indicated. *B*, Western blot analysis of the recombinant protein. *Lane M* corresponds to prestained protein molecular mass markers.

RESULTS

To understand the conformation of the N-protein and its role in the aggregation process, we felt the need to isolate the soluble N-protein, but not a refolded, which may differ in conformation. We observed that when overproduced in bacteria by inducing cells with 500 μM IPTG at 37 °C for 4 h, the N-protein composed more than 50% of the total cellular proteins. However, the majority of the overexpressed protein formed inclusion bodies, posing a problem for its purification (data not shown). We observed that BL21(DE3) cells induced at A_{600} = 0.3 with 100 μM IPTG for 14 h at 16 °C produced the majority of the N-protein in a soluble form. To maximize the amount of N-protein in a soluble form during purification, we introduced 200 mM NaCl in buffer A and washed the inclusion pellet

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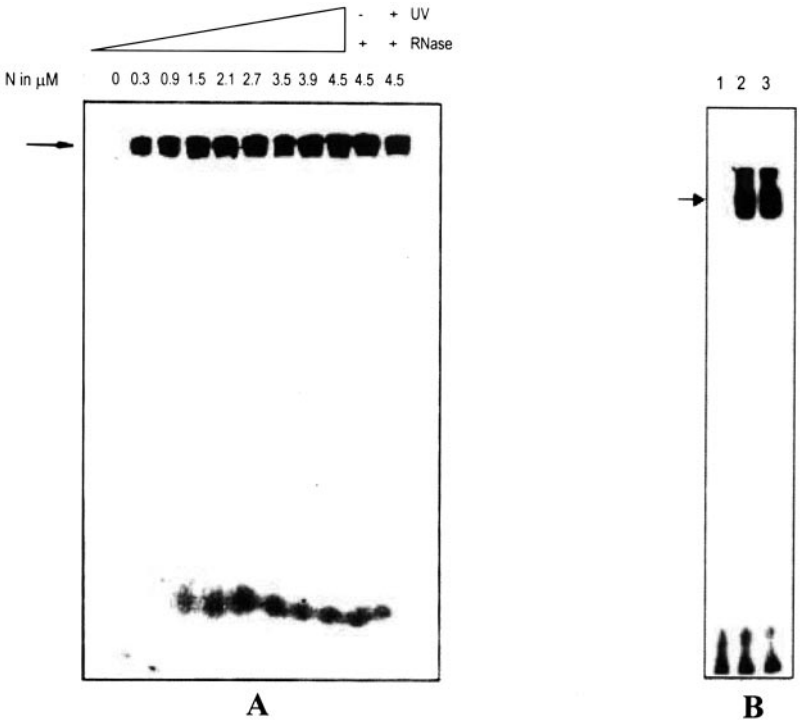

FIG. 3. *A*, ^{32}P-labeled leader RNA was incubated with the indicated amounts of N-protein (*first* through *ninth lanes*). Also shown is the preformed complex treated with RNase before (*tenth lane*) and after (*eleventh lane*) UV cross-linking. The complexes were resolved using 6% native gel. Shifted bands are indicated by *arrows*. *B*, shown is the biological activity of the I-EDANS-labeled N-protein. *Lane 1*, leader RNA; *lane 2*, leader RNA + N-protein; *lane 3*, leader RNA + I-EDANS-labeled N-protein. 2.5 μM N-protein or I-EDANS-labeled N-protein (see "Experimental Procedures") was incubated with radiolabeled leader RNA, and the complex was resolved on 6% native polyacrylamide gel. The gel was dried and autoradiographed.

several times with the same buffer. We found that the above protocol successfully produces >80% of the overexpressed protein in soluble form (Fig. 2, *A* and *B*, *lane 5*). Western blotting was performed to further verify the above results (Fig. 2*B*).

Biological Activity of the Recombinant N-protein—The major biological role of the nucleocapsid protein is to bind with the viral genome and to encapsidate it in an RNase-resistant form. We tested the affinity of the recombinant protein for viral leader RNA sequence corresponding to the 5′-end of the viral anti-genome by gel electrophoretic mobility shift assays. *In vitro* transcribed leader RNA was incubated with increasing amounts of recombinant N-protein, and the complex was resolved on 6% native polyacrylamide (see "Experimental Procedures"). We observed the appearance of a shifted band upon the addition of protein, and the intensity of the shifted band increased sharply with increasing protein concentrations, indicating a high affinity of the recombinant protein for leader RNA (Fig. 3*A*, *first* through *ninth lanes*). We next examined the ability of the recombinant protein to protect viral RNA from RNase action *in vitro*. To demonstrate that we have treated the complex formed between leader RNA and the N-protein with RNase in another set, the complex was UV-cross-linked and treated with RNase (Fig. 3*A*, *tenth* and *eleventh lanes*). The intensity of the shifted band remained the same as that of the RNase-untreated complex (Fig. 3*A*, *ninth lane*), indicating a true encapsidation of viral leader RNA by the N-protein.

Spectroscopic Studies of the N-protein Structure—CD spectra have been widely used as to monitor secondary structure. Fig. 4 shows the far-UV CD spectrum of the solubilized N-protein after removal of Triton X-100. An attempt was made to extract relative amounts of secondary structure present in the protein from the CD spectra. Although the quality of fit was not excellent, it indicated a large amount of random-coil form present. From the work of Baskakov and Bolen (9), it is clear that physiological concentrations of osmolytes can drive the folding equilibrium toward the native state. If the random-coil conformations present in the soluble N-protein reflect partial denaturation, a significant secondary structure alteration of the N-protein is anticipated upon addition of an osmolyte. So, far-UV CD spectra were measured in the presence of an osmolyte (D-sorbitol), and secondary structure formation was monitored. The addition of D-sorbitol led to some enhancement of the CD spectrum and hence secondary structure contents.

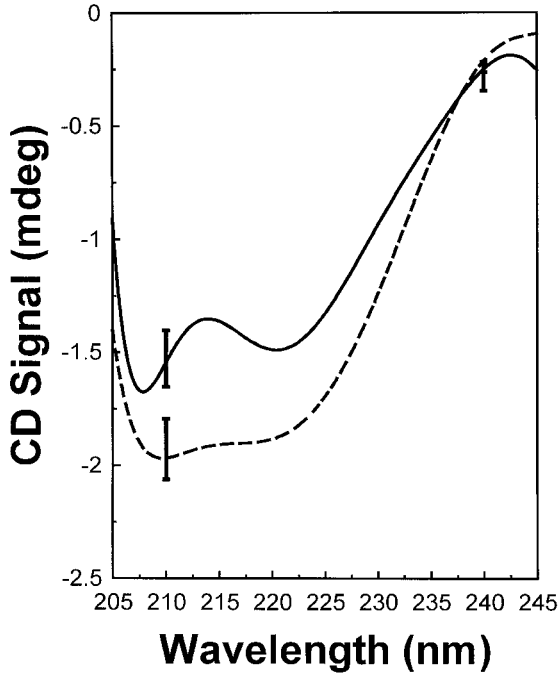

FIG. 4. **Circular dichroism spectra of the N-protein in the absence (——) and presence (– – –) of 250 mM D-sorbitol.** Measurements were carried out in 50 mM Tris-HCl (pH 8) containing 150 mM NaCl at ambient temperature. For each measurement, an average of four scans was taken. A time constant of 2 s and a scan speed of 50 nm/min were used for spectral scanning. The protein concentration was 10 μM. The path length was 1 mm. Each spectrum is an average of four independent measurements and was smoothed. *Error bars* at two ends of the spectra indicate uncertainties. *mdeg*, millidegrees.

We also attempted to measure the effect of osmolytes on N-protein structure by the use of steady-state fluorescence anisotropy. Fluorescence anisotropy is a function of probe motion. In the absence of internal probe motions, fluorescence

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TABLE I
Steady-state fluorescence anisotropy of the I-EDANS-labeled N protein in the presence and absence of osmolytes

A concentrated stock of the soluble recombinant N-protein was labeled with I-EDANS and diluted with 50 mM Tris-HCl (pH 8) containing 100 mM NaCl in the fluorometer cuvette to a final concentration of 0.5 μM. The fluorescence anisotropy of this protein sample was measured in the presence and absence of 250 mM D-sorbitol.

D-Sorbitol conc	Fluorescence anisotropy values
0	0.02
250 mM	0.05

anisotropy is a function of rotational tumbling of the whole molecule. Rapid internal motions, typical of partially or fully disordered states, reduce the anisotropy values. An increase in order generally should increase the fluorescence anisotropy value of a probe covalently attached to the protein. Sulfhydryl groups are selective attachment points for fluorescent probes. The DTNB reaction was used for determining the number of titrable sulfhydryl groups. 2.99 ± 0.1 groups reacted with DTNB, indicating that these sulfhydryl groups are available for attachment of covalent probes. 2.93 ± 0.19 I-EDANS molecules were incorporated into the protein using the protocol described under "Experimental Procedures." This I-EDANS-labeled N-protein showed full biological activity with respect to RNA-binding ability as shown in Fig. 3*B*.

Table I reports the steady-state fluorescence anisotropy values of the I-EDANS-labeled soluble recombinant N-protein in the presence and absence of the osmolyte D-sorbitol. The anisotropy value in the absence of D-sorbitol is very low compared with the expected value for a typical globular protein of 48 kDa. For example, when attached to the λ-repressor dimer (molecular mass of 52 kDa), a dansyl chloride probe gave an anisotropy value of ~0.1 and a rotational correlation time of ~20 ns under similar conditions (12). We measured the lifetimes of the attached I-EDANS probe in the presence and absence of D-sorbitol. The lifetimes were 15.1 and 15.3 ns in the absence and presence of D-sorbitol, respectively. If one calculates the rotational correlation time from the Perrin equation assuming a limiting anisotropy value of 0.4, the values are 0.8 and 2.2 ns without and with D-sorbitol, respectively. These rotational correlation times clearly indicate that there are very significant internal motions indicative of disorder. The addition of D-sorbitol led to a significant increase in the fluorescence anisotropy and rotational correlation time, indicating that the addition of osmolytes leads to a decrease in internal motions. This is consistent with the modest enhancement of the CD spectrum in the presence of osmolytes.

Self-aggregation of the N-protein—The spectroscopic studies reported above indicate that the soluble N-protein is partially denatured and disordered. It is generally believed that such partially denatured and disordered proteins are often prone to aggregation. To investigate the self-aggregating tendency of the soluble recombinant N-protein, dynamic light scattering measurements were performed. For this experiment, Triton X-100 was removed, and aggregation was monitored using dynamic light scattering. DLS studies showed that the CHPV N-protein formed large aggregates and that the change in aggregate size occurred with time. While monitoring the aggregation process of the N-protein, it was seen that 15 min after the removal of Triton X-100, the protein started to form aggregates (seen from the increasing value of the Stokes radius). Fig. 5*A* shows the DLS results after ~60 min, when the protein formed particles with an average Stokes radius of 8.8 nm (a monomer of the size of the N-protein should have a Stokes radius of ~2–3 nm). After 18 h, it reached the saturation of the

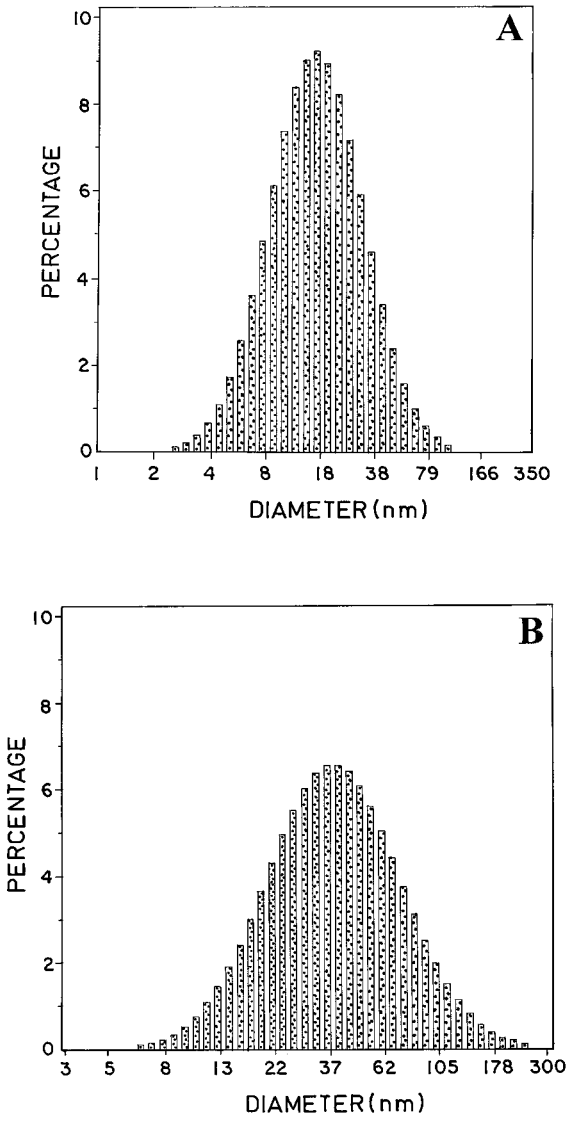

FIG. 5. *A*, distribution of particle size measured by DLS 60 min after removal of Triton X-100 from the soluble recombinant N-protein. Dynamic light scattering analysis was done using the Triton X-100-removed soluble recombinant N-protein at 1 mg/ml. *B*, distribution of particle size measured by DLS 18 h after removal of Triton X-100. Dynamic light scattering analysis was done using the Triton X-100-removed soluble recombinant N-protein at 1 mg/ml. The hydrodynamic radius was calculated using Stokes-Einstein's equation. All data were collected taking an average of 100 scans, and the experiments were done in 50 mM Tris-HCl (pH 8) containing 100 mM NaCl.

aggregation process and formed particles with an average Stokes radius of 16.05 nm (Fig. 5*B*). The standard error of these measurements was ~5% based on three independent measurements. Clearly, the soluble N-protein has a tendency to aggregate, which is reduced in the presence of nonionic detergents such as Triton X-100.

Fluorescence anisotropy indicated that the osmolyte D-sorbitol increased the local order in the N-protein. The reduction of disorder, even a local one, may have an influence on the solubility and aggregation of proteins. So we investigated the roles of osmolytes present in the cells in solubilization of the N-protein; and for this purpose, static light scattering experiments were performed. The static light scattering technique is widely used to study protein aggregation and disaggregation (13). Aggregation of proteins gives rise to high light scattering values, which reduce upon disaggregation. In this experiment, first Triton X-100 was removed from a very high concentration

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TABLE II

Aggregation of Triton X-100-C removed soluble recombinant N-protein in the presence and absence of osmolytes using static light scattering

A concentrated stock of the soluble recombinant N-protein was taken and from it was removed Triton X-100 using Bio-Beads SM-2 adsorbent. Then, the protein stock was diluted with 50 mM Tris-HCl (pH 8) containing 100 mM NaCl to a final concentration of 50 μM. Static light scattering intensity was monitored at 340 nm and excited at the same wavelength as a function of time. The same experiment was repeated, but 250 mM D-sorbitol was added during the detergent removal. All values were corrected for the appropriate blank buffer value, and they represent the scattering intensities after 30 min.

Conditions	Scattering intensities
50 μM CHPV N-protein	876.97
50 μM CHPV N-protein + 250 mM D-sorbitol	259.3

of soluble recombinant N-protein using Bio-Beads SM-2 adsorbent. The protein was then diluted to a final concentration of 50 μM in a fluorometer cuvette, and scattering values were monitored as a function of time. This same experiment was repeated, but D-sorbitol (250 mM) was included in the buffer during detergent removal and light scattering measurements. Table II reports the light scattering intensities after 30 min of 50 μM N-protein in the presence and absence of osmolytes. The light scattering value was reduced by severalfold in the presence of osmolytes. Clearly, the presence of 250 mM D-sorbitol inhibited aggregation, suggesting that even an increase in the local order leads to inhibition of self-aggregation of the N-protein.

Chaperone-like Activity of the P-protein—As seen from the above experiments, the N-protein is prone to aggregation, which is partially alleviated in the presence of biological concentrations of osmolytes. We have explored other factors that may influence the aggregation of the nascent N-protein. The general machinery of chaperones may certainly influence the outcome. Recent reports have suggested that the P-protein forms a stable complex with the N-protein (14). We have thus explored whether the P-protein can act as a chaperone for the newly synthesized N-protein. During refolding at high denaturant concentrations, many proteins form large aggregates of partially folded proteins. Many chaperones are known to suppress this kind of aggregation, which can be used as an assay for chaperone activity. Light scattering is widely used to study protein associations at equilibrium, and the scattering intensity is proportional to the weight-average molecular weight of the protein. Thus, aggregation gives rise to a high light scattering value that is reduced in the presence of many chaperones. For the purpose of this study, first the highly concentrated stock of the soluble N-protein was treated with Bio-Beads SM-2 adsorbent to remove Triton X-100. After removal of the detergent, the protein was denatured with 8 M urea and was allowed to refold by diluting in a native buffer in the fluorometer cuvette. Aggregation during refolding was monitored by light scattering intensity. The scattering values as a function of time were relatively flat, but much higher than those of the protein-alone controls. This suggests that refolding and aggregation were over within the mixing dead time. The light scattering intensities at 10 min are presented in Table III for different combinations of the P- and N-proteins.

As evident from Table III, in the presence of the P-protein, the relative scattering intensities of the N-protein decreased significantly, indicating that the CHPV P-protein can act like a chaperone and can inhibit the aggregation of the N-protein. To further substantiate the static light scattering data, DLS experiments were performed. Aggregation of the N-protein in the presence of the P-protein was monitored using dynamic light scattering. 100 μM N-protein was mixed with 100 μM P-protein;

TABLE III

Aggregation during refolding of urea-denatured N-protein in the absence and presence of varying concentrations of P-protein monitored by static light scattering

A concentrated stock of the soluble recombinant N-protein (N) was taken, and from it was removed Triton X-100 using Bio-Beads SM-2 adsorbent. After removal of the detergent, the protein was denatured with 8 M urea. This denatured stock was diluted with 50 mM Tris-HCl (pH 8) containing 100 mM NaCl to a final concentration of 2 μM in the fluorometer cuvette, and aggregation during refolding was monitored by light scattering intensity in the absence and presence of varying concentrations of P-protein (P) (1, 2, 4, and 8 μM). The values presented were corrected for their appropriate buffer values containing respective amounts of P-protein. Excitation and emission wavelengths were set at 340 nm.

Conditions	Relative scattering intensities
2 μM N	803.91
2 μM N + 1 μM P	479.41
2 μM N + 2 μM P	234.61
2 μM N + 4 μM P	199.41
2 μM N + 8 μM P	190.61

Triton X-100 in the protein mixture was removed using Bio-Beads; and aggregation was monitored. As seen previously, in the absence of the P-protein, the N-protein started to aggregate after removal of the detergent within 15 min; and after ~100 scans (after 60 min), the Stokes radius reached 8.8 nm. However, in the presence of the P-protein, there was no such increase in the Stokes radius of the particles (data not shown). Thus, the P-protein hinders the aggregation of the N-protein, supporting the static light scattering data.

To determine if the CHPV P-protein is a generalized chaperone or is specific for the N-protein only, we measured whether the CHPV P-protein can prevent the dithiothreitol-induced aggregation of insulin. We found that the P-protein (10 μM) had no influence on the dithiothreitol-induced aggregation of insulin (data not shown). These two findings suggest that the chaperone-like activity of the P-protein may be specifically directed toward the N-protein or related proteins. In a recent report, it has been suggested that osmolytes and chaperones may act in a synergistic manner to refold proteins (15). We have thus attempted to determine whether the P-protein and D-sorbitol act in a synergistic manner. Table IV shows the results of suppression of N-protein aggregation by D-sorbitol and the P-protein as measured by static light scattering. Clearly, the addition of the P-protein to D-sorbitol further enhanced aggregation suppression, over and above that achieved by the osmolyte alone. Thus, the presence of the osmolyte D-sorbitol lowers the concentration requirement of the P-protein for chaperone-like action.

If the P-protein exerts chaperone-like activity toward the N-protein specifically, it is possible that a specific structural determinant of the N-protein is recognized and not a general hydrophobic interaction. This structural determinant must be formed in the partially folded intermediates that are prone to aggregation. Thus, we have tried to detect specific N-protein/P-protein interaction. One of the best ways to study protein/protein interaction is through use of fluorescence. We used the I-EDANS-labeled CHPV N-protein as a probe and titrated it with increasing concentrations of unlabeled bacterially expressed recombinant P-protein. Fig. 6 shows the fluorescence enhancement as a function of increasing concentrations of P-protein. The enhancement saturated quickly, suggesting a specific complex formation. The fluorescence increase can be fitted to a binding equation with a dissociation constant of $(1.19 \pm 0.33) \times 10^{-6}$ M. From the previous discussion, it is quite clear that the CHPV N-protein can self-aggregate and that the CHPV P-protein can inhibit this aggregation process, and so

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TABLE IV
Aggregation during refolding of urea-denatured N-protein in the absence and presence of varying concentrations of P-protein and D-sorbitol monitored by static light scattering

A concentrated stock of the soluble recombinant N-protein was taken, and from it was removed Triton X-100 using Bio-Beads SM-2 adsorbent. After removal of the detergent, the protein was denatured with 8 M urea. This denatured stock was diluted with 50 mM Tris-HCl (pH 8) containing 100 mM NaCl to a final concentration of 1 μM in the fluorometer cuvette, and aggregation during refolding was monitored by light scattering intensity in the absence and presence of varying concentrations of D-sorbitol and in the presence of 0.25 μM each P-protein and D-sorbitol. Excitation and emission wavelengths were set at 340 nm.

Conditions	Scattering intensities
1 μM N	471
1 μM N + 125 mM D-sorbitol	324.6
1 μM N + 250 mM D-sorbitol	248.9
1 μM N + 500 mM D-sorbitol	250.2
1 μM N + 0.25 μM P	374.6
1 μM N + 125 mM D-sorbitol + 0.25 μM P	274
1 μM N + 250 mM D-sorbitol + 0.25 μM P	261.8

can D-sorbitol. We have previously seen that D-sorbitol induced some local order, which may have some influence on the binding isotherm. Fig. 6 also shows the fluorescence increase in the presence of 250 mM D-sorbitol. It is clear that the N- and P-proteins interact in the presence of the osmolyte. The data can be fitted to a dissociation constant of $(0.8 \pm 0.37) \times 10^{-6}$ M.

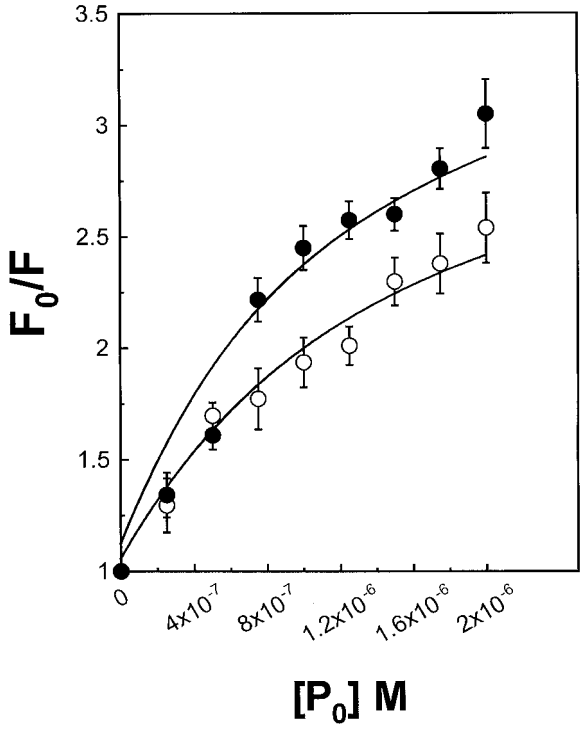

FIG. 6. **Fluorescence enhancement of the I-EDANS-labeled N-protein as a function of increasing concentrations of P-protein.** ○, relative fluorescence values in the presence of D-sorbitol; ●, relative fluorescence values in the absence of D-sorbitol. 0.5 μM I-EDANS-labeled soluble recombinant N-protein was titrated with varying concentrations of phosphoprotein P (0–2 μM), and the changes in relative fluorescence intensity were monitored at 37 °C. All values were corrected for the buffer values; and in the case of the presence of the P-protein, the values were corrected for the contribution of corresponding amounts of P-protein. These titrations were carried out in the presence and absence of 250 mM D-sorbitol in 50 mM Tris-HCl (pH 8) containing 100 mM NaCl. Each point is an average of three independent measurements.

DISCUSSION

In this study, we report for the first time the full-length cloning of the Chandipura virus N gene. To fulfill the requirement of a large amount of N-protein for a detailed structure-function analysis, it has been previously overexpressed both in prokaryotic and eukaryotic systems. Expression of the N-protein alone in COS cells (16) as well as in bacteria (17) resulted in formation of large insoluble aggregates, creating obstacles for biological studies. However, in the VSV system, bacterial coexpression of the P-protein along with the N-protein improves its solubility (18). Here we have successfully overexpressed the N-protein in bacteria by changing induction parameters, resulting in the production of a soluble monomeric form, which facilitated its characterization. We have developed a single-step rapid purification procedure for this viral product using a Mono-Q column. The N-protein encapsidates the nascent genomic RNA during viral replication. The biological activity of the soluble recombinant nucleocapsid protein is demonstrated by its ability to bind viral RNA sequences and enwrapping the leader RNA in an RNase-resistant form. Encapsidation even at very low concentrations of N-protein indicates that the recombinant protein is highly efficient in its RNA-binding ability.

A disordered and free protein may be prone to aggregation. This is also the case with the N-protein. However, what happens *in vitro* may be offset by some other factors *in vivo*, as in the case of protein folding. We have identified two very important factors that help prevent aggregation and solubilize the N-protein, thus maintaining a pool of active N-protein for viral packaging. The principal biological activity of the N-protein is to package the viral genomic RNA. For packaging of a large number of progeny viral genomes, a steady supply of N-proteins is required during the life cycle of the virus. The self-aggregation tendency of the free N-protein is of considerable importance for the study of the protein. It is, however, unlikely that self-aggregation is a property involved in the biological role of the protein. It is, however, not unlikely that the disorder of the isolated N-protein is because of its dissociation from the viral genomic RNA. Many DNA-binding proteins are known that are disordered in the free state and may have evolved that way as a requirement for binding to multiple target sites (19). It is possible that such a requirement is also present in the N-protein.

The role of osmolytes in driving protein toward the native state is now well established. This happens because, in particular, the peptide backbones have very unfavorable free energies for transfer from water to osmolyte solutions. Thus, they favor any state that hides the peptide backbone from external solvent, including the native state. The effect of osmolytes on the aggregated state is not well known. It is generally believed that aggregation occurs through partially folded states of proteins. Although, in principle, osmolytes should favor native states and thus reduce aggregation, the process is complicated by the fact that aggregation also leads to removal of exposed surface area through protein/protein interaction. It is possible that osmolyte action on aggregation is more complex and requires detailed study. In this study, we have shown that osmolytes are capable of driving the N-protein toward a more structured state that is soluble and less prone to aggregation. Clearly, at least in this case, osmolytes have a beneficial effect by disrupting the protein aggregation. Although we do not have any direct *in vivo* data concerning the role of osmolytes in the viral life cycle, given the widespread occurrence of intracellular osmolytes, it is likely that the tendency of aggregation of the N-protein will at least be partially offset by intracellular osmolytes.

The role of the P-protein in the viral life cycle is well estab-

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lished. In its phosphorylated form, the protein is a transcription activator. We have established another important role of the P-protein in the functioning of the N-protein. The recombinant P-protein interacts with the N-protein, with dissociation constants in the low micromolar range. This affinity is crucial for interaction with the N-protein and prevents aggregation. The P-protein has a highly negatively charged N-terminal domain, which is a characteristic of many chaperones. It has been hypothesized that the negatively charged regions keep the protein complexes soluble, preventing aggregation (20).

A recent report has indicated that osmolytes and chaperones together can be used to refold proteins (in this case, a mutant) that are otherwise difficult to refold. Based on the experiments reported here, we suggest that the cooperation of an osmolyte and a chaperone in refolding a protein may have a much wider occurrence *in vivo* and may be a widely applicable *in vitro* tool for the refolding of proteins.

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