Articles

Reversible and Irreversible Steps in Assembly and Disassembly of Vesicular Stomatitis Virus: Equilibria and Kinetics of Dissociation of Nucleocapsid−M Protein Complexes Assembled in Vivo[†]

Douglas S. Lyles* and Margie O. McKenzie

Department of Microbiology and Immunology, Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, North Carolina 27157

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ABSTRACT: The matrix (M) protein of vesicular stomatitis virus (VSV) condenses the viral nucleoprotein core (nucleocapsid) into a tightly coiled, helical nucleocapsid−M protein (NCM) complex. Using NCM complexes assembled in vivo, the dissociation of M protein was examined by measuring the apparent affinity constants and kinetic constants for M protein binding to NCM complexes immediately after detergent solubilization of the virion envelope. Wild-type VSV strains and viruses with mutations in their M proteins were analyzed using sedimentation and light-scattering assays. At physiological ionic strength, the binding reaction had the characteristics of a dynamic reversible equilibrium. A temperature-sensitive M protein mutant lost the ability of M protein to reversibly dissociate from the nucleocapsid, while a temperature-stable revertant regained the ability to undergo reversible dissociation. In contrast to the results obtained at physiological ionic strength, nucleocapsids stripped of M protein by incubation at high ionic strength (250 mM NaCl) were not able to bind M protein at low ionic strength with the same high affinity seen in NCM complexes assembled in vivo. The effect of incubation at 250 mM NaCl was shown to be due to a change in nucleocapsids rather than a change in soluble M protein. This result supports the idea that nucleocapsids devoid of M protein must undergo a separate step that initiates high-affinity binding of M protein in vivo.

Enveloped viruses assemble by budding from host membranes. During this process, the nucleoprotein core of the virus (nucleocapsid) binds to the cytoplasmic surface of the host membrane. Many viruses encode a matrix (M) protein that mediates binding of nucleocapsids to the host membrane. Vesicular stomatitis virus (VSV),[1] the prototype rhabdovirus, has been widely used in studies of virus assembly. The VSV nucleocapsid contains an 11 kb single strand RNA genome, 1300 copies of a single major nucleoprotein (N protein), and lesser amounts of two polymerase-associated proteins, P and L (*1*). The virion contains approximately 2000 copies of M protein, which are bound to the nucleocapsid to form a condensed, tightly coiled, helical nucleocapsid−M protein (NCM) complex that appears to give the virion its characteristic bullet-like shape (*2*−*5*).

VSV nucleocapsids devoid of M protein can be isolated by solubilizing the virion envelope with detergents in high ionic strength buffers (e.g., 250 mM NaCl) and appear to be a loosely coiled, flexible helix. At low ionic strength, the NCM complex remains intact following detergent solubilization of the envelope and retains the distinctive morphology of the internal contents of the virion (*2*−*4*). Despite the important role of M protein binding to nucleocapsids in virus assembly, relatively few studies have addressed the biochemical mechanism of M protein binding. In particular, the important biochemical question of whether M protein binding to nucleocapsids involves either reversible or irreversible processes has yet to be resolved. The experiments presented here indicate that both reversible and irreversible steps are involved in assembly of VSV M protein with nucleocapsids.

M proteins usually enter the virus assembly pathway as soluble proteins in the cytoplasm (*6*−*8*). However, purified M protein or NCM complexes have a tendency to aggregate, particularly at low or intermediate ionic strength, thus raising concerns whether the isolated components retain the native structure found in vivo. We have developed methods to

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* To whom correspondence should be addressed.

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[1] Abbreviations: G, envelope glycoprotein; L, large RNA polymerase subunit; M, matrix protein; N, nucleocapsid protein; NCM, nucleocapsid−M protein (complex); P, phosphoprotein polymerase subunit; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ts, temperature-sensitive; VSV, vesicular stomatitis virus; wt, wild-type.

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maintain the solubility of M protein and to analyze NCM complexes assembled in vivo immediately after solubilization of virion envelopes without further purification (*8*−*10*). In particular, light scattering by NCM complexes can be evaluated without further isolation, since the complex is large compared to the envelope components solubilized in detergent micelles (*10*). Light scattering is very sensitive to the amount of M protein bound to the nucleocapsid. The experiments presented here used a combination of sedimentation and light-scattering assays to test the reversibility of M protein dissociation from NCM complexes as a function of ionic strength. At physiological ionic strength, the binding reaction had the characteristics of a dynamic reversible equilibrium. In contrast to the results obtained at physiological ionic strength, nucleocapsids stripped of M protein by incubation at high ionic strength (250 mM NaCl) were not able to bind M protein with the same high affinity seen in NCM complexes assembled in vivo. The effect of incubation at 250 mM NaCl was shown to be due to a change in nucleocapsids rather than a change in soluble M protein. A strong case has been made that nucleocapsids incubated with 250 mM NaCl retain the native structure of nucleocapsids found in infected cells (*1*−*3*). Thus the results presented here support the idea that nucleocapsids must undergo a separate step that initiates high-affinity binding of M protein in vivo. This may explain why intracellular nucleocapsids do not associate with M protein in infected cells, despite the high intracellular concentration of M protein, until virus assembly has been initiated at the host plasma membrane.

EXPERIMENTAL PROCEDURES

Purification of Virions and M Protein. Radiolabeled wild-type (wt) VSV (San Juan and Orsay strains, Indiana serotype), the temperature-sensitive (ts) mutant tsO23, and its temperature-stable revertant tsO23r1 were prepared by growth in BHK cells in the presence of [^{35}S]methionine, purified by sucrose gradient centrifugation, and assayed for protein concentration and specific radioactivity as described (*9*). The purified virus was dialyzed overnight against 10 mM Tris, pH 7.8, at 4 °C and used immediately without freezing and thawing. M protein was solubilized from purified virus in buffer containing 1% Triton X-100 and 0.25 M NaCl and was purified by ion-exchange chromatography as described previously (*8*).

Sedimentation Analysis of M Protein Binding to Nucleocapsids. ^{35}S-labeled VSV at the indicated concentration was incubated in buffer containing 10 mM Tris, pH 8.1, 0.1% Triton X-100, and the indicated amount of NaCl at room temperature (23 ± 1 °C). Samples were centrifuged in a Beckman Airfuge, and supernatants and pellets were analyzed by SDS−PAGE and quantitated as described (*9*). The fraction of total M protein bound to nucleocapsids (*b*) was calculated as the amount of M protein in the pellet divided by that in the pellet plus supernatant.

The stoichiometry of M protein binding to nucleocapsids in virions is approximately a 2:1 molar ratio to the major nucleocapsid protein N. The simplest case would be if each N protein molecule were able to bind two M protein molecules independently of the remaining N protein in the nucleocapsid. In this case, two different models must be considered: the binding reaction is bimolecular, i.e., one M molecule binds to one of two independent sites on N protein, or trimolecular, i.e., two M molecules interact with one binding site on N protein. When the data were fit to the bimolecular model in which the two binding sites on N protein were allowed to have independent binding affinities, the data were best fit if these two affinities were the same (not shown). For simplicity in routine analysis, each of the two binding sites on N protein was assumed to have the same affinity and is represented in reaction 1 by $N_{1/2}$:

$$MN_{1/2} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} M + N_{1/2} \qquad \text{(reaction 1)}$$

The data were analyzed as the fraction of M protein bound to nucleocapsids (*b*) as a function of the total M protein concentration (M_t), calculated as 30% of the total virion protein concentration (*10*). The concentrations of the reactants are $[MN_{1/2}] = M_t b$, $[M] = M_t(1 - b)$, and $[N_{1/2}] = 2N_t - M_t b = M_t\,[(2N_t/M_t) - b]$, where N_t is the total concentration of N protein. Thus the equation for the dissociation constant would be

$$K_1 = \frac{k_1}{k_{-1}} = \frac{[M][N_{1/2}]}{[MN_{1/2}]} = \frac{M_t(1-b)\left(\frac{2N_t}{M_t} - b\right)}{b} \qquad \text{(eq 1)}$$

Similarly the trimolecular reaction is represented by reaction 2:

$$M_2N \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} 2M + N \qquad \text{(reaction 2)}$$

In this case $[M_2N] = M_t b/2$, $[M] = M_t(1 - b)$, and $[N] = N_t - (M_t b/2) = (M_t/2)[(2N_t/M_t) - b]$, and the dissociation constant would be

$$K_2 = \frac{k_1}{k_{-1}} = \frac{[M]^2[N]}{[M_2N]} = \frac{M_t^2(1-b)^2\left(\frac{2N_t}{M_t} - b\right)}{b} \qquad \text{(eq 2)}$$

The values of *b* as a function of M_t were fit to eq 1 or 2 using a nonlinear least squares fit (JMP statistical analysis software, SAS Institute, Inc., Cary, NC) of one parameter, either K_1 or K_2.

Stopped-Flow Analysis. Time courses for the changes in light-scattering intensity of NCM complexes upon shifting the concentration of NaCl were determined using an Applied Photophysics DX.17MV stopped-flow spectrophotometer at a wavelength of 500 nm and the detector at a 90° angle to the light path. NCM complexes in buffer containing 10 mM NaCl and 0.1% Triton X-100 were prepared as described above and placed in the sample syringe. The second syringe contained the same buffer with twice the desired final NaCl concentration. Equal volumes from each syringe were injected into the mixing chamber, and 400 data points were collected using a split time base. The initial 200 data points were collected from either 0 to 2 s or 0 to 5 s to determine the fast phase ($t_{1/2}$ = 0.5−2 s), which corresponds to dissociation of M protein from NCM complexes (*10*), and 200 data points were collected in the subsequent 100 s to determine the slower phase ($t_{1/2}$ = 20−30 s), which corre-

sponds to reversal of the self-association of NCM complexes that occurs upon removal of M protein. In most of the experiments, the slow phase only accounted for 10%−20% of the total change in light-scattering intensity. A total of four reaction traces were collected at each NaCl concentration. The data obtained at final NaCl concentrations of 60 and 120 mM were fit to either a single exponential, $I(t) = I_a e^{-k_a t} + I_f$, or to a double exponential, $I(t) = I_a e^{-k_a t} + I_b e^{-k_b t} + I_f$, by a nonlinear least squares fit of three parameters (I_a, k_a, I_f) or five parameters (I_a, k_a, I_b, k_b, I_f), respectively, using software supplied by the manufacturer. A single exponential was assumed to fit the data unless the normalized variance was reduced by at least a factor of 2 by assuming a double exponential. In most cases where a double exponential was found, the improvement in the fit reduced the variance by a factor of 5−10. The data obtained at a final NaCl concentration of 250 mM NaCl were not fit well using exponential decays. Instead, the data from 0−4 s were fit to either a linear steady state model, $I(t) = I_0 - mt$, or a linear steady state with a pre-steady-state exponential phase, $I(t) = I_a e^{-k_a t} + I_0 - mt$, by a linear least squares fit of two parameters (I_0 and m) or a nonlinear least squares fit of four parameters (I_a, k_a, I_0, m), respectively, where m is the slope and I_0 is the y-intercept of the linear phase. The slopes were expressed as a percent change in light-scattering intensity per second, calculated as $m/[I(0) - I(f)]$, where I(0) and I(f) were the initial and final light scattering intensities at $t = 0$ and $t =$ 105 s, respectively.

Interpretation of Rate Data. Light-scattering intensity is affected by the concentration, molecular weight, and shape of the light-scattering species. The effects of each of these factors on light scattering by NCM complexes have been explored in detail previously (*10*). The effects of the shape changes that occur upon shifting the ionic strength are relatively minor and occur rapidly (within the first 100−200 ms) and thus could be effectively ignored in the present study. Most of the change in light-scattering intensity of NCM complexes upon shifting the ionic strength arises from the dissociation of M protein, which has two effects. One is the reduction in molecular weight of the NCM complexes, and the other is the reduction in effective protein mass concentration that contributes to light scattering, since the monomeric M protein (26 kDa molecular weight) essentially "disappears" compared to the remaining NCM complexes (90−140 MDa) (*10*). Thus the effective protein concentration (C) that contributes to light-scattering intensity is $C_N + bC_M$, where C_N and C_M are the mass concentrations (g/mL) of N protein and M protein initially present in the NCM complex, respectively. Likewise, the molecular weight of the NCM complex (MW) can be expressed in terms of C_N and C_M as $(C_N + bC_M)/N_{NC}$, where N_{NC} is the concentration of nucleocapsids in mol/mL. Thus the light-scattering intensity can be expressed as a function of the fraction of M protein bound in the NCM complex, b, as

$$I = KC(\mathrm{MW}) = \frac{K}{N_{NC}}(C_N + bC_M)^2 \qquad \text{(eq 3)}$$

where K is a constant that gives the light-scattering intensity of nucleocapsids per unit concentration and molecular weight and incorporates experimental constants such as wavelength, detector angle, and refractive index. In stopped flow experiments in which M protein dissociation is exponential, $b(t) = (1 - b_f)e^{-k_a t} + b_f$, where k_a is the apparent rate constant for the re-equilibration (relaxation) and b_f is the final equilibrium value of b. Substituting into eq 3 gives

$$I(t) = \frac{K}{N_{NC}}[C_N{}^2 + 2C_N C_M b_f{}^2] + \frac{K}{N_{NC}}[(2C_M{}^2 b_f + 2C_N C_M) \times (1 - b_f)]e^{-k_a t} + \frac{K}{N_{NC}}[C_M{}^2 (1 - b_f)^2]\, e^{-2k_a t} \qquad \text{(eq 4)}$$

Thus the quadratic term from eq 3 gives rise to two exponentials, one with an apparent rate constant that is the same as that of the M protein relaxation (k_a) and another that is $2k_a$. In practice, the second exponential was rarely detected, presumably because it is much smaller in magnitude, since $(1 - b_f)^2 < (1 - b_f)$ and $C_M{}^2 \ll (2C_M{}^2 b_f + 2C_N C_M)$. Similarly, data fit to a linear steady state model are described by $b(t) = 1 - at$ for $t = 0$ to $1/a$, where a is the linear dissociation rate as a fraction of M protein molecules per second. Substituting into eq 3 gives

$$I(t) = \frac{K}{N_{NC}}[C_N{}^2 + 2C_N C_M + C_M{}^2] - \frac{K}{N_{NC}}[2C_N C_M + 2C_M{}^2]at + \frac{K}{N_{NC}}[C_M{}^2]a^2 t^2 \qquad \text{(eq 5)}$$

Since $C_M{}^2 \ll 2C_N C_M + 2C_M{}^2$, the quadratic term has little effect on the linear decay of light-scattering intensity until late time points, at which time the steady state assumption also breaks down. Therefore, linear rates were determined using the initial 60%−75% of the decay curve. Also, if the quadratic term is not significant, $I(0) - I$(f) is approximately equal to $(K/N_{NC})(2C_N C_M + 2C_M{}^2)$, and the slope of the change in light-scattering intensity, m, can be used to determine $a = m/[I(0) - I(f)]$.

The rate constants, k_a, for re-equilibration of M protein binding following a shift in NaCl concentration to 60 or 120 mM were used together with the measured equilibrium constants to calculate k_1 and k_{-1} using classical relaxation theory such as that used in temperature-shift experiments (*11*). For the bimolecular model (reaction 1), $k_1 = k_a/[1 + (2M_e/K_1)]$, where M_e is the new equilibrium concentration of free M protein. For the trimolecular reaction model (reaction 2), a similar derivation (not shown) gives $k_1 = k_a/[1 + (12N_e{}^2/K_2)]$.

RESULTS

Concentration Dependence of M Protein Dissociation from NCM Complexes at Physiological Ionic Strength. M protein in VSV virions is bound to the nucleocapsid in an approximately 2:1 molar ratio of M protein to the major nucleocapsid protein, N (*1*). M protein dissociates from the viral nucleocapsid at high ionic strength (≥250 mM) following detergent solubilization of the virion envelope, while solubilization of the envelope at physiological ionic strength results in partial dissociation. It has not been clear whether dissociation of M protein from the nucleocapsid represents a state of reversible equilibrium between bound and unbound M protein or results from an irreversible

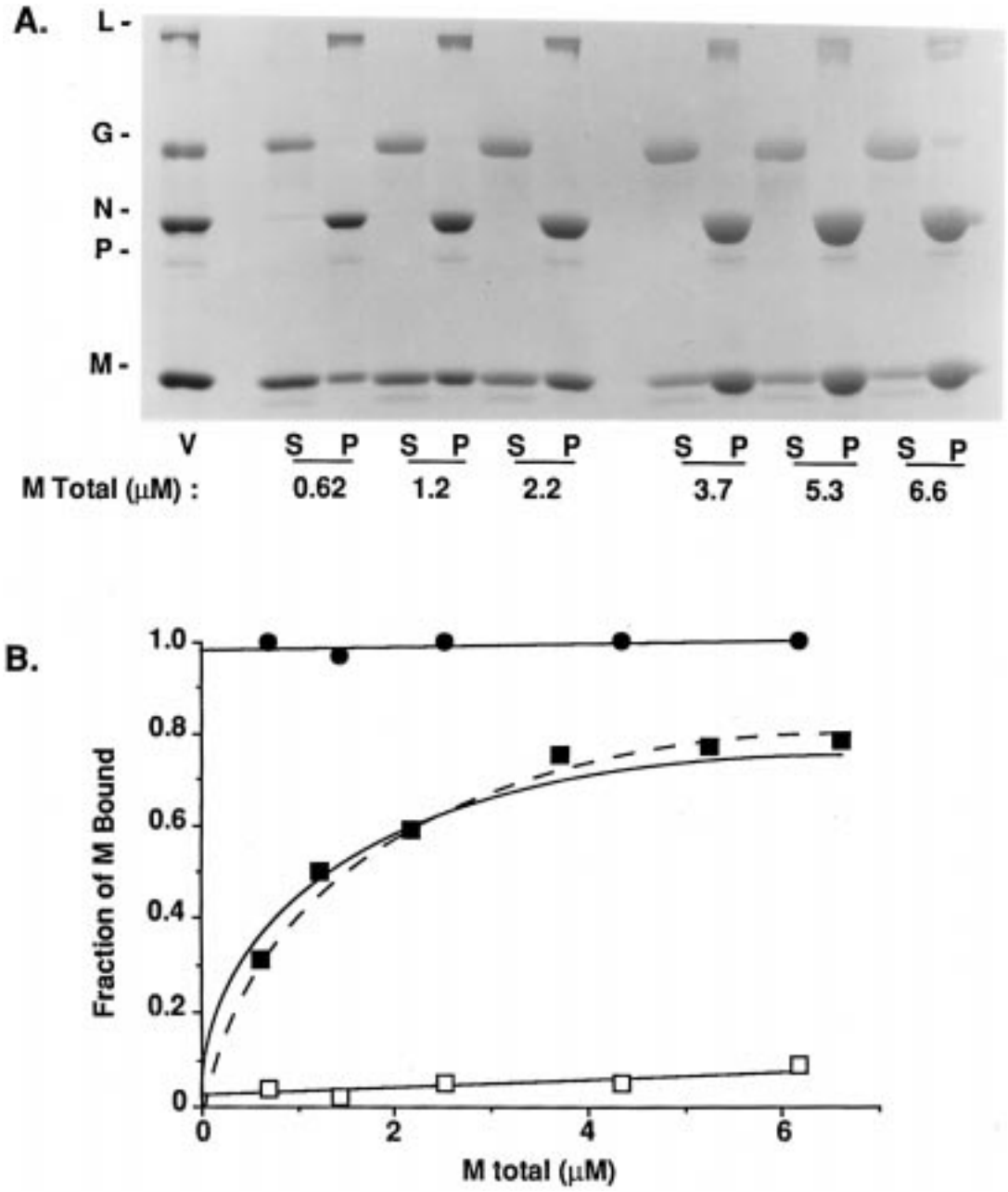

FIGURE 1: Concentration dependence of M protein dissociation from NCM complexes. (A) ^{35}S-labeled VSV was mixed with unlabeled VSV to give the indicated total concentration of M protein. Virus envelopes were solubilized in buffer containing 0.1% Triton X-100 and 120 mM NaCl. Samples were centrifuged in a Beckman Airfuge, and supernatants (S) and pellets (P) were analyzed by SDS−PAGE, a fluorograph of which is shown. V = a marker lane of unfractionated virions. (B) The fraction of total M protein bound to NCM complexes was determined as a function of protein concentration by densitometry of data similar to Figure 1A obtained at NaCl concentrations of 10 mM (closed circles), 120 mM (closed squares), and 250 mM (open squares). Data obtained at 120 mM NaCl were fit to the equilibrium binding equation for a bimolecular reaction (solid curves) and for a trimolecular reaction (dashed curves).

dissociation that does not go to completion at physiological ionic strength.

If M protein binding results from a reversible equilibrium, then the concentration dependence of M protein binding should be governed by reaction 1 or 2 (Experimental Procedures section) and should vary in a manner predictable by the laws of mass action. The concentration dependence of M protein binding to nucleocapsids was evaluated immediately after solubilization of virion envelopes without further purification to minimize the perturbation of NCM complexes assembled in vivo. The concentrations of M protein and nucleocapsids were varied in parallel by varying the initial concentration of virions prior to solubilization of the envelope. In the experiment shown in Figure 1A, purified VSV labeled with [^{35}S]methionine (54 μg/mL total viral protein, 0.62 μM total M protein) was mixed with increasing amounts of nonradiolabeled VSV to yield a total M protein concentration that ranged from 0.62 to 6.6 μM. Virion envelopes were solubilized with 0.1% Triton X-100 in the presence of 120 mM NaCl and incubated for 20 min at room temperature to allow equilibration of M protein binding. The amount of M protein bound to the nucleocapsid was analyzed by pelleting the nucleocapsids and analyzing the supernatant and pellet by SDS−PAGE and fluorography. As expected, the pellet fractions contained the major nucleocapsid protein, N, as well as the two polymerase-associated

Table 1: Equilibrium and Rate Constants for Dissociation of NCM Complexes Assembled in Vivo[a]

[NaCl] (mM)	pH	dissociation constant, K_1 (μM)	relaxation rate constant, k_a (s^{-1})	off-rate k_1 (s^{-1})	on-rate k_{-1} (s^{-1} μM^{-1})
60	8.1	0.087 ± 0.050	0.49 ± 0.21	0.14	1.6
120	8.1	0.41 ± 0.17	0.32 ± 0.09	0.17	0.42
	7.0	0.34 ± 0.11	−		
	6.5	0.35 ± 0.12	−		
	6.0	0.45 ± 0.21	−		
	5.5	0.30 ± 0.01	−		

[a] Dissociation constants were determined following solubilization of virion envelopes with Triton X-100 at the indicated [NaCl] and pH as in Figure 1, except that the protein concentration was varied to include at least 50% of the binding isotherm. Fraction of total M protein in NCM complex versus protein concentration was fit to eq 1 (bimolecular model) to determine K_1. Data shown are means ± range for two determinations. Relaxation rate constants were determined from changes in 90° light-scattering intensity following solubilization of virion envelopes with Triton X-100 at 10 mM NaCl, then shifting to the indicated final [NaCl] in a stopped-flow spectrophotometer as in Figure 2. The total M protein concentration was 0.26 μM. Values for k_1 and k_{-1} were calculated from k_a and K_1. Data shown are means ± SD for 16 determinations.

proteins, P and L, while the supernatant contained the transmembrane envelope glycoprotein, G (Figure 1A). The amount of radiolabeled M protein bound to the nucleocapsid increased progressively from the lowest concentration (0.62 μM) to the highest (6.6 μM). This is the behavior expected for reversible complex formation in which the lower concentration of reactants favors dissociation of the complex. In some experiments, a small amount of a proteolytic fragment of M protein was generated during the incubation after solubilization of the envelope (presumably by minor contamination with cellular proteases), visible in Figure 1A as a faint band below the M protein band. As shown previously, this protein does not bind to nucleocapsids and was not included in the calculation of M protein binding (*9*).

Figure 1B shows densitometric analysis of M protein binding to nucleocapsids at 120 mM NaCl in the experiment in Figure 1A (closed squares). Also shown are data from control samples incubated at 10 mM NaCl, which prevents M protein dissociation (closed circles), and at 250 mM NaCl, at which maximum dissociation of M protein occurs (open squares). The concentration dependence of M protein binding at 120 mM NaCl was well-described by equations for both the bimolecular reaction (reaction 1: One M molecule binds to one of two independent sites on N protein) as well as the trimolecular reaction (reaction 2: two M molecules interact with one binding site on N protein). Nonlinear least squares analysis was used to fit the data to either reaction 1 (Figure 1B, solid curve) or reaction 2 (dashed curve). However, the data were not sufficiently precise to distinguish these two models.

The apparent dissociation constants obtained at 120 mM NaCl as well as at 60 mM NaCl and at different pH values in repeated experiments similar to those in Figure 1 are listed in Table 1. The apparent affinity of M protein binding to nucleocapsids was enhanced by approximately 4-fold upon reducing the ionic strength from 120 to 60 mM. However, binding of M protein to nucleocapsids was not notably affected by pH in the range pH 5.5−8.1 (Table 1).

Kinetics of dissociation of NCM complexes at physiological ionic strength is exponential while that at 250 mM NaCl are nonexponential. The kinetics of M protein dissociation upon shifting the concentration of NaCl was determined by changes in light-scattering intensity in a stopped-flow spectrophotometer in order to calculate the apparent rate constants k_1 and k_{-1} in reaction 1 or 2. Measuring the changes in light-scattering intensity is a sensitive method to detect dissociation of M protein from NCM complexes as a result of a shift in ionic strength (*10*). Dissociation of M protein converts the NCM complex (135 MDa molecular mass) into a mixture of nucleocapsids (90 MDa molecular mass) and soluble, monomeric M protein (26 kDa molecular mass) (*8*). Since light-scattering intensity is proportional to the molecular weight and concentration of the scattering particle, the soluble M protein does not contribute significantly to scattering intensity compared to nucleocapsids or NCM complexes. Similarly, the solubilized envelope components, such as the envelope glycoprotein and lipids, do not contribute significantly to the light-scattering intensity due to the small size of the detergent micelles in which they reside, thus making it unnecessary to purify NCM complexes prior to analyzing their dissociation (*10*).

The data shown in Figure 2A are from stopped-flow analysis of the changes in 90° light-scattering intensity for NCM complexes upon shifting the NaCl concentration from 10 to 60 mM (curve a), 120 mM (curve b), or 250 mM (curve c). It has been shown previously that most of the change in scattering intensity is due to the change in molecular mass and effective protein concentration following dissociation of M protein, which occurs over a few seconds. Depending on their concentration, NCM complexes have a tendency to self-associate to a greater extent than nucleocapsids lacking M protein. A slower change in intensity ($t_{1/2}$ > 10 s) results from reversal of this self-association. At the protein concentration used in the experiment shown in Figure 2, the slow phase accounted for about 10%−20% of the total change in light-scattering intensity. The data were analyzed by nonlinear least squares fit to exponential decays (smooth curves in Figure 2A). The data obtained at 60 and 120 mM NaCl (curves a and b) were fit well by assuming two exponential decays, the first corresponding to the dissociation of M protein and the second corresponding to reversal of the self-association of NCM complexes. The quality of the fit is shown by a plot of the differences between the experimental and fitted curves (residuals) at the bottom of Figure 2A, which were randomly distributed around the *x*-axis for curves a and b.

The apparent rate constants (k_a) for the dissociation of M protein in curves a and b (Figure 2A) were used together with the equilibrium constants (K_1) to derive k_1 and k_{-1} in reaction 1 by classical relaxation theory (*11*) and are shown in Table 1 for M protein dissociation at 60 and 120 mM NaCl. These data show that the differences in binding affinities at the two NaCl concentrations were primarily due to differences in on-rate (k_{-1}). The conclusion was the same if k_1 and k_{-1} were calculated for the trimolecular reaction model (reaction 2) (not shown).

In the case of the shift of NCM complexes from 10 to 250 mM NaCl (Figure 2A, curve c), the kinetic data were well-described by exponential decays only at later time points ≥5 s. However, at time points <5 s, there was a marked

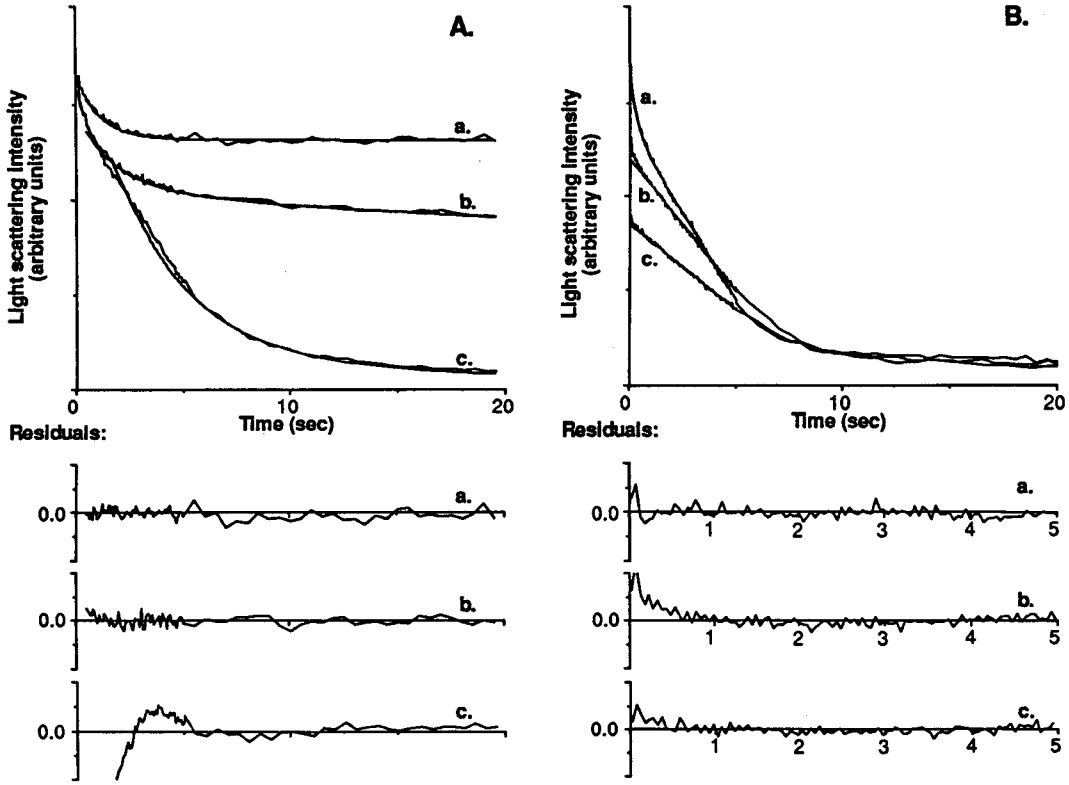

FIGURE 2: Kinetics of dissociation of NCM complexes. (A) NCM complexes (0.6 μM total M protein) in buffer containing 10 mM NaCl and 0.1% Triton X-100 were injected into the mixing chamber of a stopped-flow apparatus together with an equal volume of buffer to give a final NaCl concentration of 60 mM (a), 120 mM (b), or 250 mM (c). Light-scattering intensity was recorded as a function of time after mixing. The data were fit to a double exponential, $I(t) = I_a e^{-k_a t} + I_b e^{-k_b t} + I_f$, by a nonlinear least squares fit (smooth curves). Also shown are plots of the differences between the experimental and fitted curves (residuals) on an expanded scale. (B) NCM complexes (0.6 μM total M protein) in buffer containing 10 mM (a), 60 mM (b), or 120 mM (c) NaCl and 0.1% Triton X-100 were injected into the mixing chamber of a stopped flow apparatus together with an equal volume of buffer to give a final NaCl concentration of 250 mM. Light-scattering intensity was recorded as a function of time after mixing. The data from 0−5 s were fit to either a linear steady state model (b and c), $I(t) = I_0 - mt$, by a linear least squares fit, or a linear steady state with a pre-steady-state exponential phase (a), $I(t) = I_a e^{-k_a t} + I_0 - mt$, by a nonlinear least squares fit (smooth curves). Also shown are plots of the differences between the experimental and fitted curves (residuals) on an expanded scale.

deviation of the experimental points from the fitted curve. The fit was not improved by assuming additional exponential terms in the fitting equation (not shown), indicating that the mechanism of M protein dissociation at 250 mM NaCl includes processes that cannot be accounted for by simple exponentials. The theoretical considerations that predict an exponential decay assume that the perturbation of the binding equilibrium upon shifting the ionic strength is small (*11*), which is certainly not the case here. Thus it is possible that additional nonexponential terms contribute to the rate of M protein dissociation due to the failure of the assumptions of the relaxation theory. This hypothesis was tested by decreasing the extent of the perturbation of the binding equilibrium. In the experiment shown in Figure 2B, NCM complexes were preincubated in buffer containing 10 mM (curve a), 60 mM (curve b), or 120 mM NaCl (curve c) prior to shifting to 250 mM NaCl and analyzing light-scattering intensity in the stopped-flow apparatus. In all three cases, the data could not be adequately described by a series of exponentials. In fact, curve c showed the greatest deviation from exponential fits of the data even though the perturbation of the binding equilibrium was smallest.

Inspection of the data in Figure 2B indicated that curve c was nearly linear for time points ≤5 s and that curves a and b might contain linear regions between approximately 1 s and 5 s preceded by a short (<1 s) exponential phase. This behavior would be typical of a reaction containing kinetic intermediates, in which the linear phase reflects a steady state accumulation of the intermediate and the initial exponential phase reflects the approach to steady state. The simplest such model would be reaction 3:

$$MN_{1/2} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} M + N_{1/2} \longrightarrow M' + N'_{1/2} \qquad \text{(reaction 3)}$$

In this model, the intermediate species M and $N_{1/2}$, retain sufficient affinity for each other that reversibility of binding makes a significant contribution to the rate equation. The species M′ and $N'_{1/2}$ represent states of the M and N proteins that have little binding affinity so that the dissociation is effectively irreversible. This difference between M and M′ or N and N′ proteins could result, for example, from alternative conformational states. Similar models in which only one of the two components (either M protein or N protein) undergoes an irreversible change give similar results. The data between 0 and 5 s in Figure 2B were fit to a steady state model (reaction 3). The fit for curve a included an exponential pre-steady-state term followed by a linear steady state phase, while curves b and c were fit only to the linear steady state equation. All three curves were well-described by the steady state model as shown by the random distribution of the residuals around the *x*-axis. There was slight

Table 2: Steady State and Pre-Steady-State Rates of Dissociation of NCM Complexes in the Presence of 250 mM NaCl[a]

initial [NaCl] (mM)	pH	steady state rate (% of total/s)	pre-steady-state amplitude (% of total)	pre-steady-state rate constant (s^{-1})	*N*
120	8.1	9.0 ± 1.9	−	−	8
60		12.3 ± 1.0	−	−	8
10		12.0 ± 2.0	17.6 ± 2.7	3.30 ± 1.29	15
10	7.5	16.8 ± 2.0	17.4 ± 1.7	2.80 ± 0.69	6
	7.1	13.4 ± 1.7	19.0 ± 0.7	2.57 ± 0.26	6
	6.6	8.49 ± 2.19	25.0 ± 1.6	2.12 ± 1.28	6
	6.1	3.17 ± 0.10	36.1 ± 0.7	1.95 ± 0.04	4
	5.5	3.35 ± 0.11	35.1 ± 2.0	1.73 ± 0.17	4

[a] VSV virion envelopes were solubilized at the indicated initial NaCl concentrations and pH. The NaCl concentration was shifted to 250 mM in a stopped-flow spectrophotometer and 90° light-scattering intensity was measured as a function of time after the shift. Data from 0−4 s were fit to a linear steady state rate with a pre-steady-state exponential phase by a nonlinear least squares fit. Data shown are means ± SD for *N* determinations.

deviation of curves b and c from the fit at times <0.5 s. This deviation was due to the small amount of pre-steady-state phase that was not included in these fits.

The slopes of the linear portion of curves similar to those in Figure 2B are shown in Table 2 as well as the pre-steady-state terms for curves similar to curve a. The slopes are expressed as a percent change in scattering intensity per second, calculated by dividing the fitted slopes by the total change in light-scattering intensity. The data in Table 2 show that when expressed in this way, these rates were nearly the same regardless of whether the NCM complexes were partially dissociated by preincubation at 60 or 120 mM NaCl prior to the shift to 250 mM NaCl. This behavior is consistent with the steady state model, which predicts that the rate of dissociation of NCM complexes should be approximately proportional to the initial concentration of NCM complexes, which in turn is related to the total change in light-scattering intensity. This prediction was further confirmed by analyzing the dissociation over a 15-fold concentration range (from 2−30 μg/mL total viral protein) in which there was no significant change in the kinetics of dissociation (not shown).

Dissociation of M Protein from Nucleocapsids at 120 mM NaCl is Reversible Whereas Dissociation at 250 mM NaCl Is Not. The kinetic data in Figure 2 indicate that M protein dissociation from nucleocapsids at 120 mM NaCl was consistent with a simple reversible equilibrium (eq 1 or 2), while dissociation at 250 mM NaCl required inclusion of an additional step to give a steady state model consistent with the data. The data were well-described by the model in eq 3, in which the additional step described by k_3 is irreversible. Further support for this model was obtained in the experiments shown in Figure 3, which directly tested the reversibility of M protein dissociation from nucleocapsids. Nucleocapsids and M protein dissociated at high ionic strength were reassembled into NCM complexes by dialysis to lower the concentration of NaCl to 10 mM. The behavior of the reassembled NCM complexes at different NaCl concentrations was evaluated by subjecting them to a second round of dissociation in which the kinetics was determined by stopped-flow analysis. Purified ^{35}S-labeled virions were diluted into buffer containing 0.1% Triton X-100 and either 120 or 250 mM NaCl or 10 mM NaCl as a negative control for undissociated complexes. At the protein concentrations used (50 μg/mL total protein, 0.6 μM total M protein), M protein dissociation would be approximately 80% at 120 mM and approximately 95% at 250 mM NaCl (Figure 1). NCM complexes were reassembled by dialysis

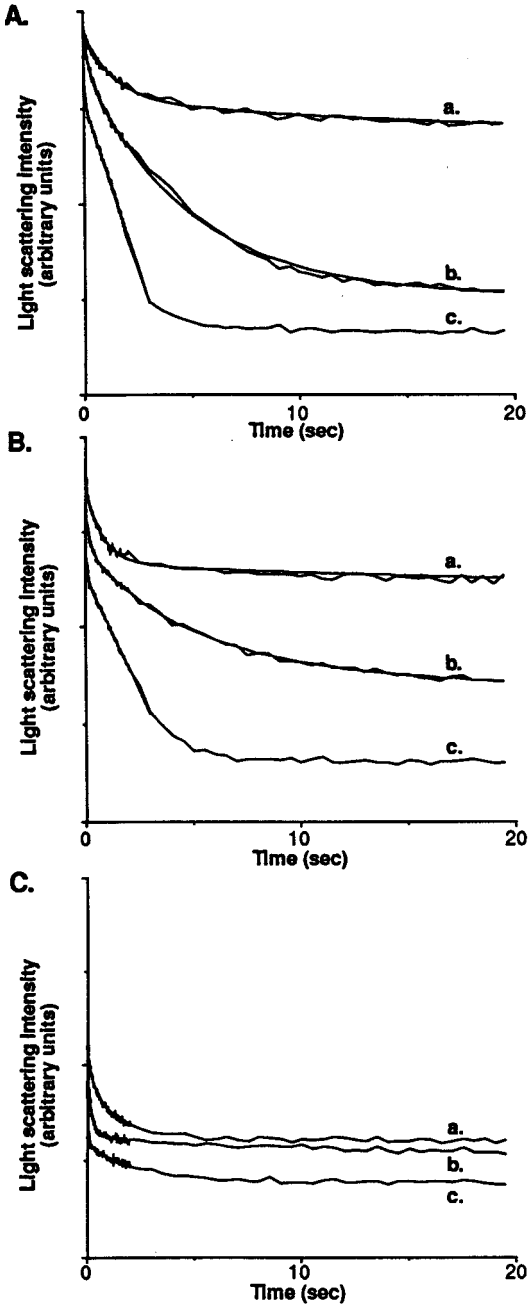

FIGURE 3: Kinetics of dissociation of NCM complexes reassembled in vitro. Virion envelopes were solubilized in buffer containing 0.1% Triton X-100 and either 10 mM (A), 120 mM (B), or 250 mM (C) NaCl. Samples (0.6 μM total M protein) were dialyzed for 2 h against 10 mM Tris, 10 mM NaCl, 1 mM dithiothreitol, 0.1% Triton X-100 at 4 °C. Conductivity measurements were used to assess completeness of NaCl equilibration. The resulting NCM complexes reassembled in vitro were injected into the mixing chamber of a stopped-flow apparatus together with an equal volume of buffer to give a final NaCl concentration of 60 mM (a), 120 mM (b), or 250 mM (c). Light-scattering intensity was recorded as a function of time after mixing. In A and B, the data obtained at 60 and 120 mM NaCl were fit to a double exponential while those obtained at 250 mM NaCl from 0 to 4 s were fit to a linear steady state with a pre-steady-state exponential phase by a nonlinear least squares fit (smooth curves).

Table 3: Rate Constants for Dissociation of Reassembled NCM Complexes[a]

[NaCl] (mM)	complexes analyzed	relaxation rate constant, k_a (s^{-1})	steady state rate (% of total/s)
60	NCM-10	0.60 ± 0.12	
	NCM-120	1.02 ± 0.23	
	NCM-250	0.8 ± 0.37	
	M alone	0.033 ± 0.005	
120	NCM-10	0.16 ± 0.04	
	NCM-120	0.20 ± 0.03	
	NCM-250	4.8 ± 1.5	
	M alone	0.085 ±0.013	
250	NCM-10		17 ± 7
	NCM-120		15 ± 3
	NCM-250	19 ± 8	
	M alone	0.42 ± 0.04	

[a] VSV virion envelopes were solubilized at NaCl concentrations of 10 mM (NCM-10), 120 mM (NCM-120), or 250 mM (NCM-250), and the samples were dialyzed against buffer containing 10 mM NaCl to reassemble the NCM complexes. Purified soluble M protein was also dialyzed against buffer containing 10 mM NaCl to induce the formation of M protein aggregates (M alone). The NaCl concentration was shifted to the indicated NaCl concentration in a stopped-flow spectrophotometer and 90° light-scattering intensity was measured as a function of time after the shift. Data were fit to either an exponential decay to determine the relaxation rate constant, k_a, or to a linear steady state rate with a pre-steady-state exponential phase by a nonlinear least squares fit. Data shown are means ± SD for 16 determinations.

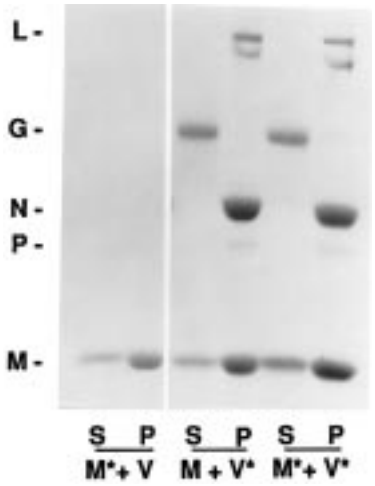

FIGURE 4: Exchange of soluble M protein with endogenous M protein in NCM complexes. M protein solubilized from virus in buffer with 1% Triton X-100 and 250 mM NaCl was purified by ion-exchange chromatography and mixed with intact virions. Virus envelopes were solubilized, and supernatant and pellet fractions were analyzed as in Figure 1A. M* + V = ^{35}S-labeled M protein + nonradiolabeled virions, M + V* = nonradiolabeled M protein + ^{35}S-labeled virions, M* + V* = ^{35}S-labeled M protein + ^{35}S-labeled virions.

for 2 h against buffer containing 10 mM NaCl. Reassociation of M protein with nucleocapsids was confirmed by sedimentation and analysis of the pellets and supernatants by SDS−PAGE similar to the experiments in Figure 1 (data not shown). The reassembled NCM complexes were shifted to NaCl concentrations of 60, 120, or 250 mM, and light-scattering intensity was analyzed in the stopped-flow apparatus (Figure 3). The rate data derived from these experiments are summarized in Table 3.

The results obtained with negative control (undissociated) NCM complexes (Figure 3A) as well as those reassembled after dissociation at 120 mM NaCl (Figure 3B) were similar to those obtained immediately after solubilization of the virus envelope (Figure 2). In particular, shifting the NaCl concentration to 60 or 120 mM (curves a and b) led to partial dissociation of the NCM complexes that was well-described by exponential decays with rate constants similar to those obtained previously (compare Tables 1 and 3). Also, shifting the NaCl concentration to 250 mM (curves c) led to complete dissociation by a nonexponential process that appeared to consist of a linear steady state phase preceded by a brief pre-steady-state exponential phase. These results indicate that reassociation of M protein and nucleocapsids that had been partially dissociated at 120 mM NaCl led to complexes indistinguishable from the original NCM complexes assembled in vivo, based on binding kinetics and equilibria.

In contrast to the results obtained following dissociation of NCM complexes at 120 mM NaCl, reassembly of NCM complexes following dissociation at 250 mM NaCl resulted in formation of complexes with strikingly different properties from those of NCM complexes assembled in vivo. Shifting the NaCl concentration to 60, 120, and 250 mM NaCl all led to nearly complete dissociation of the reassembled complexes (Figure 3C), and the rate constants for the dissociation (Table 3) were approximately an order of magnitude faster than those of NCM complexes assembled in vivo. Furthermore, dissociation of the reassembled complexes after shifting the NaCl concentration to 250 mM no longer displayed the steady state character of NCM complexes assembled in vivo. Instead, the dissociation was well-described by an exponential decay with a rate constant of approximately 20 s^{-1}.

An underlying assumption in these experiments is that the M protein had in fact reassociated with nucleocapsids. Alternatively, M protein might have self-associated into M protein aggregates approximately the same size as nucleocapsids. Such aggregates have been shown to form at low ionic strength in the absence of nucleocapsids (*8*, *12*). To address this possibility, M protein aggregates were formed in the absence of nucleocapsids, and the kinetics of dissociation following a shift in ionic strength was determined. M protein purified from virions was dialyzed against buffer containing 10 mM NaCl in the absence of nucleocapsids to induce the formation of M protein aggregates. The resulting aggregates were then shifted to NaCl concentrations of 60, 120, or 250 mM, and light-scattering intensity was analyzed in the stopped-flow apparatus. The rate constants derived from these experiments are included in Table 3 for comparison with the kinetics of dissociation of NCM complexes. The behavior of M protein aggregates differs considerably from that of NCM complexes assembled either in vivo or in vitro, since the rate constants for dissociation of M protein aggregates are considerably slower.

Exchange of Solubilized M Protein with Endogenous M Protein in NCM Complexes. The data in Figure 3 indicate that upon dissociation at 250 mM NaCl, either M protein or nucleocapsids (or both) undergo changes that are not readily reversed and prevent high-affinity binding upon reducing the NaCl concentration. The irreversible step at 250 mM NaCl involves a change in the nucleocapsids and not M protein, as shown by the data in Figure 4. An exchange experiment was used to demonstrate that radiolabeled M protein solubilized at 250 mM NaCl was competent to bind nucleo-

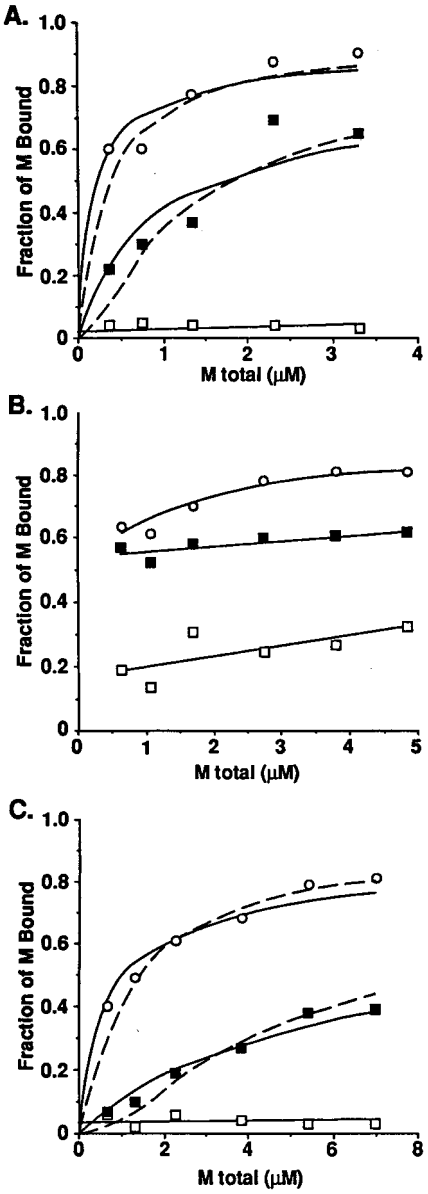

FIGURE 5: Dependence of M protein dissociation from NCM complexes on virus strain. The fraction of total M protein bound to NCM complexes was determined as a function of protein concentration by densitometry of data similar to Figure 1A obtained at NaCl concentrations of 60 mM (open circles), 120 mM (closed squares), and 250 mM (open squares): (A) wt Orsay strain. (B) tsO23 virus. (C) tsO23r1 virus.

capsids at 120 mM NaCl by its ability to exchange with nonradiolabeled endogenous M protein in NCM complexes. M protein was solubilized from purified virus labeled with [^{35}S]methionine in buffer containing 1% Triton X-100 and 250 mM NaCl and was purified by ion-exchange chromatography. In the experiment shown in Figure 4, purified ^{35}S-labeled M protein (0.4 μM) was mixed with unlabeled virions (M* + V) prior to solubilization of the envelope (total M protein concentration = 1.8 μM), and its distribution between the supernatant and pellet was evaluated. As controls, purified nonradiolabeled M protein was mixed with ^{35}S-labeled virions (M + V*) to determine the distribution of M protein originating from virions, and ^{35}S-labeled purified M protein was mixed with ^{35}S-labeled virions (M* + V*) to determine the distribution of total labeled M protein. In all three samples approximately 65% of the labeled M protein

Table 4: Equilibrium and Rate Constants for Dissociation of NCM Complexes of Wild-Type, Temperature Sensitive, and Revertant VSV Strains[a]

virus	[NaCl] (mM)	dissociation constant, K_1 (μM)	relaxation rate constant, k_a (s^{-1})	off-rate k_1 (s^{-1})	on-rate k_{-1} (s^{-1} μM^{-1})
wt Orsay	60	0.13 ± 0.03	0.48 ± 0.07	0.13	1.0
tsO23r1		0.46 ± 0.08	1.14 ± 0.14	0.54	1.18
wt Orsay	120	0.68 ± 0.17	0.15 ± 0.05	0.081	0.12
tsO23r1		5.8 ± 1.2	0.39 ± 0.11	0.34	0.06

[a] Dissociation constants were determined following solubilization of virion envelopes with Triton X-100 at the indicated [NaCl] and pH as in Figure 1, except that the protein concentration was varied to include at least 50% of the binding isotherm. Fraction of total M protein in NCM complex versus protein concentration was fit to eq 1 (bimolecular model) to determine K_1. Data shown are means ± range for two determinations. Relaxation rate constants were determined from changes in 90° light-scattering intensity following solubilization of virion envelopes with Triton X-100 at 10 mM NaCl and then shifting to the indicated final [NaCl] in a stopped-flow spectrophotometer as in Figure 2. The total M protein concentration was 0.39 μM (wt Orsay) and 0.40 μM (tsO23r1). Values for k_1 and k_{-1} were calculated from k_a and K_1. Data shown are means ± SD for 16 determinations.

was present in the pellet, as determined by densitometry of the fluorograph shown in Figure 4. As additional controls, in a separate experiment it was confirmed that >85% of the purified M protein remained in the supernatant in the absence of nucleocapsids as described previously (*8*) and that nucleocapsids that had been incubated at 250 mM NaCl were not able to bind soluble M protein at 120 mM NaCl (not shown). The results of these experiments demonstrate that purified soluble M protein exchanges freely with the M protein from virions bound to nucleocapsids. This supports the conclusion drawn from the data in Figures 1 and 3 that M protein binding to nucleocapsids at physiological ionic strength results from a reversible equilibrium. More importantly, these data indicate that M protein solubilized at 250 mM NaCl is competent to bind nucleocapsids at 120 mM NaCl. Therefore, the irreversible change that prevents reassembly of NCM complexes after dissociation at 250 mM NaCl occurs in the nucleocapsids, and not M protein.

Effect of Temperature Sensitive Mutation on Reversibility of M Protein Binding to Nucleocapsids. While the interaction of wt M protein with nucleocapsids at physiological ionic strength has the properties of a reversible equilibrium, a ts M protein mutant was subjected to irreversible processes that led to NCM complexes that were resistant to dissociation at high ionic strength. Ts M protein mutants are defective in the assembly of M protein into NCM complexes when grown at the nonpermissive temperature (39 °C) (reviewed in ref 5). However, previous results have suggested that the ts M protein is altered in its ability to associate with internal virion components even when grown at the permissive temperature (31 °C) (reviewed in ref 14). Experiments similar to that in Figure 1A were used to determine the apparent dissociation constants for M protein binding to nucleocapsids of different wt and ts VSV strains (Figure 5 and Table 4): the wt Orsay strain (Figure 5A), a ts M protein mutant of the Orsay strain (tsO23, Figure 5B), and a temperature-stable revertant of tsO23 virus (ts23r1, Figure 5C).

The primary sequence of the M protein from the Orsay strain differs from that of the San Juan strain used in Figures 1−4 by 5 out of 229 amino acids (*13*). These sequence

differences had little or no effect on the ability of the two M proteins to bind to their respective nucleocapsids (Figure 5A), since they had similar apparent dissociation constants (bimolecular) of around 0.5 μM at 120 mM NaCl (Tables 1 and 4). In contrast to the M proteins from wt virus strains, binding of M protein from tsO23 virus to nucleocapsids was clearly not a reversible equilibrium (Figure 5B). While the dependence on ionic strength generally resembled that of wt strains, there was little or no concentration dependence of M protein binding at 120 mM NaCl (closed squares, Figure 5B). Also, M protein dissociation at 250 mM NaCl did not proceed to completion at any protein concentration examined (open squares, Figure 5B). These data indicate that irreversible processes contributed significantly to the binding reaction. This may involve conversion of the ts M protein to a form incapable of dissociation (NCM′ in reaction 4) or conversion of the soluble ts M protein to a form that no longer binds nucleocapsids (M′ in reaction 4), or both processes may occur:

$$\begin{array}{ccc} MN_{1/2} & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & M + N_{1/2} \\ \downarrow k_3 & & \downarrow k_2 \\ MN'_{1/2} & & M' + N'_{1/2} \end{array} \qquad \text{(reaction 4)}$$

The virions used in these experiments were assembled at the permissive temperature for virus growth (31 °C) and analyzed at room temperature (23 °C) and thus had never been exposed to the inactivating conditions of the nonpermissive temperature for virus growth (39 °C). These results are consistent with earlier data indicating that the ts mutation alters the interaction of the M protein with the virus core components even at the permissive temperature (reviewed in ref 14).

The primary sequence of M protein from tsO23 virus (*13*) differs from that of the wt Orsay strain by three amino acid substitutions (G21E, L111F, and H227Y). In the temperature-stable revertant, tsO23r1, Phe at position 111 has reverted to Leu, but the other two substitutions are unchanged from those of tsO23 virus (*13*). The reversion at position 111 in M protein from tsO23r1 virus restored the reversible character of the M protein-nucleocapsid binding equilibrium as shown by its concentration dependence (Figure 5C), but the binding affinity was reduced approximately 5−10-fold (Table 4), due to the presence of the other two substitutions compared to M protein from wt Orsay strain. The loss of reversibility of M protein dissociation from nucleocapsids in tsO23 virus and its reversion in the tsO23r1 virus correlates with the ability of these proteins to direct virion assembly (*13*). This supports the idea that the reversible nucleocapsid−M protein binding equilibrium is important for virus assembly. Defective virus assembly at the nonpermissive temperature may result from conversion to the irreversible state(s) more rapidly at higher temperature.

Figure 6 shows the kinetics of dissociation of NCM complexes from tsO23 virus and the tsO23r1 revertant virus upon shifting the NaCl concentration from 10 to 60, 120, or 250 mM. The behavior of the NCM complexes from the temperature-stable revertant virus (Figure 6A) was similar to that of wt, but the off-rate (k_1) was increased approximately 4-fold (Table 4), due to the presence of the other

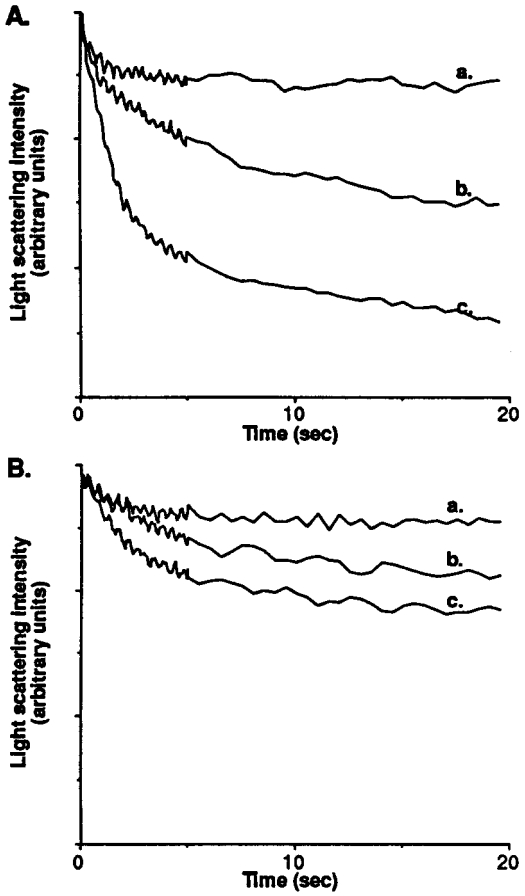

FIGURE 6: Kinetics of dissociation of NCM complexes of tsO23 and tsO23r1 viruses. NCM complexes of tsO23r1 (A) or tsO23 (B) viruses (0.8 μM total M protein) in buffer containing 10 mM NaCl and 0.1% Triton X-100 were injected into the mixing chamber of a stopped- flow apparatus together with an equal volume of buffer to give a final NaCl concentration of 60 mM (a), 120 mM (b), or 250 mM (c). Light-scattering intensity was recorded as a function of time after mixing. The *y*-axis scales in A and B are the same.

two substitutions compared to M protein from wt Orsay strain. In contrast, NCM complexes from tsO23 virus dissociated slowly, and the dissociation was incomplete even at 250 mM NaCl (Figure 6B). These data are consistent with the idea that NCM complexes from the temperature sensitive virus undergo irreversible processes that cause them to become progressively resistant to dissociation (k_3 in reaction 4).

DISCUSSION

The data presented here show that binding of M protein to nucleocapsids involves a mixture of reversible and irreversible processes. At ionic strengths near the physiological range, the binding reaction of M protein and nucleocapsids is dominated by a dynamic reversible equilibrium. The evidence for reversibility includes the concentration dependence of M protein dissociation from NCM complexes (Figures 1 and 5), the ability of exogenous soluble M protein to exchange with endogenous M protein in NCM complexes assembled in vivo (Figure 4), and the ability of M protein that had dissociated from nucleocapsids at 120 mM NaCl to reassociate with nucleocapsids following dialysis against 10 mM NaCl (Figure 3B). However, an irreversible change in the M protein-nucleocapsid interaction occurred following incubation of NCM complexes at 250

mM NaCl. Upon return to 10 mM NaCl by dialysis, M protein bound to nucleocapsids again, but with much lower apparent affinity than in NCM complexes that had not been exposed to high ionic strength (Figure 3C). Purified, soluble M protein that was originally dissociated from the nucleocapsid in the presence of 250 mM NaCl was able to exchange with M protein in NCM complexes at 120 mM NaCl with the same high affinity as the endogenous M protein (Figure 4). Thus the irreversible changes at high ionic strength only occur in nucleocapsids and not M protein. This change in the properties of nucleocapsids was also reflected in a change in the kinetics of M protein dissociation at high ionic strength to a mechanism involving an initial reversible phase followed by an irreversible step (Figure 2).

While the interaction of wt M protein with nucleocapsids at physiological ionic strength has the properties of a reversible equilibrium, ts M protein was subject to irreversible processes that led to NCM complexes that were resistant to dissociation at high ionic strength (Figures 5 and 6). A similar process was also observed with wt virus upon prolonged incubation. For example, storage of purified virus at 4 °C for approximately 1 week or at −70 °C for a matter of months led to preparations in which NCM complexes were progressively resistant to dissociation at high ionic strength (unpublished results). Thus the process leading to resistance to dissociation is likely to be a form of inactivation of the native properties of M protein.

The reversibility of M protein binding to nucleocapsids is probably critical for the early stages of infection following virus penetration into a new host cell, in which the NCM complex must dissociate in order for viral gene expression from the nucleocapsid to commence. Virus penetration into host cells occurs by a process of receptor-mediated endocytosis followed by fusion of the virus envelope with the endosome membrane, which releases the internal virion components into the cytoplasm of the cell. Following virus envelope fusion, the M protein from incoming virions has been observed to dissociate from the nucleocapsid and to be diffusely distributed in the host cell (*15*). Dissociation of M protein from nucleocapsids is presumed to be necessary to initiate infection, since M protein generally inhibits viral transcription (*14*). On the basis of the data presented here, this dissociation would be expected to occur spontaneously within a few seconds after virus penetration in response to the lack of M protein in the newly infected cell. This spontaneous mechanism would contrast with that proposed to occur for influenza viruses, in which a low pH enhances dissociation of the matrix (M1) protein from nucleocapsids (*16*). This low pH is provided in the endosomal compartment by the viral M2 ion channel protein, which makes the virus envelope permeable to protons (reviewed in reference 17). Unlike influenza viruses, VSV does not appear to contain an ion channel protein (*14*), and reduction in pH to the range found in endosomes (pH 5.5−6.0) does not enhance dissociation of the VSV M protein from nucleocapsids (Table 1).

The binding affinity of M protein for nucleocapsids is also critical for the process of virus assembly. Are the equilibrium and rate constants at physiological ionic strength measured here consistent with the intracellular concentrations of M protein and the rates of virion assembly in infected cells? Virus assembly begins around 2 h postinfection and reaches a maximum rate around 4−6 h postinfection. During this time, the intracellular M protein concentration rises from about 10^{-6} M to 3×10^{-5} M (*18*). This concentration would thus be sufficient to drive assembly of NCM complexes given a dissociation constant on the order of 1 μM. While there is currently no estimate for how rapidly NCM complexes are assembled in vivo, equilibration times for M protein binding to nucleocapsids on the order of seconds as described here would be reasonable, since pulse−chase experiments have shown that M protein appears in virions within a few minutes after its synthesis (*7*).

The nature of the change in the M protein−nucleocapsid interaction that occurs after incubation at high ionic strength is of considerable interest because of its implications for the mechanism of virus assembly. One of the paradoxes in the assembly process is that M protein and nucleocapsids are both present at high concentration in the cytoplasm of infected cells and would be expected to associate with each other. However, the only place in infected cells where they are observed to be colocalized is at the sites of budding (*19*−*21*). Thus one or both of these components must undergo an additional step that initiates the assembly process at the host plasma membrane. The step that initiates assembly of intracellular nucleocapsids with M protein in vivo may be the process that is reversed by incubation of virion nucleocapsids at high ionic strength in vitro. Alternatively, the change in nucleocapsids that occurs at 250 mM NaCl may be unrelated to the steps involved in virus assembly. However, based primarily on ultrastructural data, a strong case has been made that nucleocapsids incubated at 250 mM NaCl retain the native structure of nucleocapsids found in infected cells (*1*−*3*). Also, nucleocapsids isolated after removal of M protein in 250 mM NaCl resemble intracellular nucleocapsids in their activity in transcription and replication by the viral RNA polymerase, further supporting the idea that they are analogous to intracellular nuclecapsids involved in virus assembly (*14*).

What is the nature of the irreversible change in nucleocapsids at high ionic strength? One possibility is that the nucleocapsid protein undergoes conformational changes at high ionic strength that reduce its affinity for M protein. Another possibility is that a minor nucleocapsid component, which is irreversibly dissociated at high ionic strength, is required to initiate high-affinity assembly of M protein. This could be either another viral protein, such as P or L, or M protein in a proposed alternative, membrane-binding conformation (*22*), or even a host factor that might be present in virions in minor amounts. Future experiments will be directed toward identifying the requirements for binding of M protein to nucleocapsids with the high affinity seen in NCM complexes assembled in vivo.

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