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Thermal stability of matrix protein from Newcastle disease virus

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ABSTRACT

The thermal stability of the matrix protein (M protein) of Newcastle disease virus (NDV) has been investigated using high-sensitivity differential scanning calorimetry (DSC) at pH 7.4. The thermal folding/unfolding of M protein at this pH value is a reversible process involving a highly cooperative transition between folded and unfolded monomers with a transition temperature (T_m) of 63 °C, an unfolding enthalpy, $\Delta H(T_m)$, of 340 kcal mol^{-1}, and the difference in heat capacity between the native and denatured states of the protein, ΔC_p, of 5.1 kcal K^{-1} mol^{-1}. The heat capacity of the native state of the protein is in good agreement with the values calculated using a structure-based parameterization, whereas the calculated values for the hypothetical fully-unfolded state of the protein is higher than those determined experimentally. This difference between the heat capacity of denatured M protein and the heat capacity expected for an unstructured polypeptide of the same sequence, together with the data derived from the heat-induced changes in the steady-state fluorescence of the protein, indicates that the polypeptide chain maintains a significant amount of residual structure after thermal denaturation.

1. Introduction

Newcastle disease virus (NDV) is a negative-strand RNA virus that is enveloped. It belongs to the family of *Paramyxoviridae* (Genus *Avulavirus*) and causes respiratory and disseminated disease in poultry, depending on the strain, and has led to huge economic losses worldwide [1]. The virus genome encodes for six structural proteins, three of which are associated with the viral membrane the viral membrane derived from the host cell membrane, although the P gene also encodes a second protein, the non-structural V protein, by mRNA editing during viral transcription [2]. Two of the envelope proteins (HN and F) are transmembrane, intrinsic, glycoproteins forming spikes protruding from the lipid bilayer and play crucial roles in virus entry into the cell. HN protein, with hemagglutinin and neuraminidase activities, is the viral attachment protein that recognizes and binds to sialic acid-containing compounds [3]. F protein promotes fusion between viral and host cell membranes [4].

The other membrane-associated protein in NDV, the non-glycosylated M protein (a monomer of approximately 40 kDa), contains hydrophobic sequences, allowing the protein to interact with the inner leaflet of the viral membrane, but is not long enough to span the membrane and is a non-intrinsic membrane protein [5]. This protein is highly polymerized and forms a shield underneath the inner leaflet of the lipid bilayer of the viral membrane. It appears to play the role of gathering all of the viral components at the plasma membrane of the infected cell, leading to virion budding [5]. It has also been implicated in regulating transcription [5]. Also, M protein interacts both with nucleocapsid proteins and with the cytoplasmic tails of membrane-bound viral glycoproteins [5,6]. The M protein thus appears to be the central organizer of morphogenesis in paramyxoviruses in general [7,8] and it is believed to be involved in virus assembly in all paramyxoviruses [5].

The role of the M protein in regulating RNA synthesis in paramyxoviruses has also been studied [9–11]. However, the specific role of M protein in the viral cycle steps and the underlying molecular mechanisms remain obscure. To shed some light on this issue we have been able to show that this protein plays a key role

in implementing the domain-driven mechanism of budding, which suffices to control the shape of proteolipid vesicles during budding [12].

Abbreviations: APS, ammonium persulfate; ASA, accessible surface area; DSC, differential scanning calorimetry; NDV, Newcastle disease virus; TEMED, N,N,N′,N′-tetramethyl-ethylenediamine.

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Recently, the structures of four matrix proteins of viruses belonging to the order *Mononegavirales*–have been determined: Ebola virus [13]; respiratory syncytial virus [14]; Borna virus [15] and NDV [16]. These structures are built of one or two domains that have similar β-sandwich folds, suggesting gene duplication during evolution. The NDV M protein has more than 20% sequence identity with other paramyxovirus M proteins, including measles, mumps and the parainfluenza viruses [16] but, surprisingly, it has no sequence homology to any known protein with a similar overall structure. Furthermore, to date no data are available on the physicochemical properties that may explain the particular behaviour of M protein in the membrane of the virion, thus accounting for the interest in this kind of characterization. It has been described that M protein aggregates itself [17,18], and that aggregation is inversely correlated with ionic strength [19]. In particular, an important aspect of protein structure and function – that is, the conformational stability of M protein – has not been characterized to date. This issue is of paramount importance to understand the intermolecular recognition process (oligomerization), as well as intramolecular recognition events (monomer folding) [20–22]. Accordingly, here we used differential scanning calorimetry (DSC) together with intrinsic fluorescence to determine the structural and thermodynamic stability of NDV M protein. The results clearly show that the heat-induced denaturation of M protein is a highly cooperative, two-state reversible process in which only intact and denatured monomers are populated appreciably along the reaction. We also note that the polypeptide chain maintains some residual structure after thermal denaturation.

2. Materials and methods

2.1. Materials

Analytical grade ammonium persulphate (APS), bovine serum albumin (BSA), Coomassie Blue, EDTA, HEPES, SDS, N,N,N′,N′-tetramethyl-ethylendiamine (TEMED), Tris–HCl and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and were used without further purification. Electrophoresis and Bradford reagents were from Fermentas (UK). All other reagents were of the highest purity available. The water used for preparing the solutions was double-distilled and then subjected to a de-ionisation process.

2.2. Protein production

The lentogenic strain of NDV *LaSota* was grown at 37 °C for 48 h in the allantoic cavity of 11-day-old specific-pathogen-free chick embryos. The allantoic fluid was harvested and the virus was pelleted at 15,000 *g* for 2 h in a JLA-16.250 rotor (Beckman) at 4 °C [19,23,24]. The pellet thus obtained was re-suspended in 10 mM Tris–HCl buffer, pH 7.4, containing 100 mM NaCl, 1 mM EDTA. This solution was left at 4 °C overnight. Then, it was subjected to gentle sonication in a Branson B30 sonicator three times for 1 min each with intervals of 1 min. The virus suspension was then layered onto a continuous 10–50% (w/v) potassium tartrate gradient (in the same Tris–HCl buffer) and centrifuged at 80,000 *g* at 4 °C for 7 h in a SW-28 rotor (Beckman). The virus band was collected from the tubes and centrifuged again at 100,000 *g* at 4 °C for 90 min in a 70Ti rotor (Beckman) with at least 6 ml of 10 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA buffer, pH 7.4, helping the purified virus to precipitate.

The pellet was then re-suspended in 10 mM Tris–HCl buffer, pH 7.4, containing, 5 mM $CaCl_2$ and the mixture was left overnight at 4 °C. In the next step, KCl was added up to 1 M salt concentration. The envelopes were solubilized by adding 10% Triton X-100 (v/v) and incubating with shaking at room temperature for 30 min. Following this, the solution was centrifuged at 190,000 *g* at 4 °C for 90 min in a SW-40 Ti rotor (Beckman) and hence the nucleocapsids were pelleted and the envelopes were contained in the supernatant. This supernatant was dialyzed against 2.5 L of 10 mM Tris–HCl buffer, pH 7.4, containing, 5 mM $CaCl_2$ for 36 hours with at least 5 changes. At low ionic strength, M protein was not soluble and precipitated in the dialysis membrane while the F and NH proteins remained together in the supernatant. The content of the dialysis membrane was centrifuged at 7000 *g* at 4 °C for 30 min in a JA-20 rotor (Beckman), and the pellet containing M protein was re-suspended in 20 mM HEPES, 0.2 mM EDTA and 1 M KCl at pH 7.4 (*buffer 1*), where M protein became soluble. M protein at concentrations lower than 50 μM was stored at 4 °C until it use.

The purity of the M protein was determined by SDS-PAGE, as described in [25], on a Bio-Rad minigel device, using a flat block with a 15% polyacrylamide concentration, and also by gel-filtration, performed using a Superdex 200 10/30 HR column connected to an AKTA-purifier system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Electrophoretic conditions and Coomassie brilliant blue R-250 staining were as recommended by the manufacturers. The standards used were from a dual colour calibration kit from Fermentas UK Ltd, Cambridge, UK.

Protein concentrations were determined spectrophotometrically, using an extinction coefficient value of 27.5 mM^{-1} cm^{-1} at 280 nm [http://web.expasy.org/protparam/].

2.3. Intrinsic fluorescence

Steady-state fluorescence measurements were performed on a Hitachi F-4010 spectrofluorimeter (Hitachi Co., Ltd. Tokyo, Japan). Excitation was performed at 294 nm (with excitation and emission slit widths of 3 nm). The fluorescence measurements of M protein were carried out on protein solutions with an optical density of less than 0.3 at 280 nm to avoid the inner filter effect. All emission spectra were corrected for instrumental spectral sensitivity. The position of the middle of a chord, drawn at the 80% level of maximum intensity, was taken as the position of the emission maximum (λ_{max}). Fluorescence spectra were analyzed on the basis of the model of discrete states of tryptophan (see, for details, [26]). Measurements of pH-dependent changes in protein fluorescence were performed by downward or upward titration of the protein solution from an initial pH of 7.0, adjusting by means of a polyethylene rod moistened with either 0.1 M HCl or 0.1 M NaOH. The temperature dependence of the fluorescence spectral characteristic was measured using thermostatically-controlled water circulating in a hollow brass cell-holder. Sample temperature was monitored with thermocouple immersed in the cell under observation. The emission spectra were collected at the desired temperatures over the entire temperature range.

2.4. Differential scanning calorimetry

DSC experiments were performed on a MicroCal MC-2D differential scanning microcalorimeter (MicroCal Inc., Northampton, MA) with cell volumes of 1.22 mL, as described previously [27–29]. Before measurements, samples were degassed with gentle stirring in an evacuated chamber for 10 min at room temperature and then immediately loaded into the calorimetric cell. The reference cell was filled with the degassed final dialysis buffer. A pressure of 1.8 atm of dry nitrogen was always kept over the liquids in both cells throughout the scans in order to prevent any degassing during heating. A background scan collected with buffer in both cells was subtracted from each scan. The reversibility of the thermal transitions was verified by checking the reproducibility of the calorimetric trace in a second heating of the same sample