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Structure of the nucleocapsid-binding domain from the mumps virus polymerase; An example of protein folding induced by crystallization

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Summary

The human pathogen mumps virus, like all paramyxoviruses, encodes a polymerase responsible for virally directed RNA synthesis. The template for the polymerase is the nucleocapsid, a filamentous protein-RNA complex harbouring the viral genome. Interaction of the polymerase and the nucleocapsid is mediated by a small domain tethered to the end of the P protein, one of the polymerase subunits. We report the X-ray crystal structure of this region of mumps virus P (the nucleocapsid binding domain, or NBD, amino acids 343-391). The mumps P NBD forms a compact bundle of three α -helices within the crystal, a fold apparently conserved across the *Paramyxovirinae*. In solution, however, the domain exists in the molten globule state. This is demonstrated through application of differential scanning calorimetry, circular dichroism spectroscopy, NMR spectroscopy, and dynamic light scattering. While the mumps P NBD is compact, and has persistent secondary structure, it lacks a well-defined tertiary structure under normal solution conditions. It can, however, be induced to fold by addition of a stabilizing methylamine cosolute. The domain provides a rare example of a molten globule that can be crystallized. The structure that is stabilized in the crystal represents the fully folded state of the domain, which must be transiently realized during binding to the viral nucleocapsid. While the intermolecular forces that govern the polymerase-nucleocapsid interaction appear to be different in measles, mumps and Sendai viruses, for each of these viruses, polymerase translocation involves the coupled binding and folding of protein domains. In all cases we suggest this will result in a weak-affinity protein complex with a short lifetime, which allows the polymerase to take rapid steps forward.

Keywords

Rubulavirus; X-ray crystallography; Molten globule; TMAO

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Introduction

Mumps is an acute, but usually benign, infection occurring most often in children and young adults. Although vaccination has made it uncommon in the developed world, deficiencies in the vaccine have led to a resurgence of the disease, with recent epidemics occurring in both North America and the United Kingdom ¹.

Mumps virus is a member of the Paramyxovirus family. It is enveloped, and carries a single stranded RNA genome 15384 nucleotides in length, with the viral genes arranged in linear sequence. The nucleocapsid protein (N) packages the genomic RNA into a filamentous complex termed the nucleocapsid. In the cytoplasm of an infected cell, the viral RNA polymerase uses the nucleocapsid as the template for both transcription and genome replication (reviewed in ^{2, 3; 4}). Unencapsidated RNA cannot act as a template for the polymerase.

The Polymerase has two components, the Large protein and the Phosphoprotein (P). The Large protein is responsible for the catalytic activities of the polymerase, while the P protein performs a variety of accessory functions, including mediating direct interactions with the viral nucleocapsid. The P protein is predominantly unstructured, which is essential for its function ⁵. A centrally located coiled-coil domain serves to oligomerize P ⁶, while the C-terminus of P is involved directly in nucleocapsid-binding.

The sub-family of viruses we are concerned with is the *Paramyxovirinae*, in which there are five recognized genera. This taxonomic group includes mumps virus (genus Rubulavirus), measles virus (genus Morbillivirus) and Sendai virus (genus Respirovirus), as well as the novel zoonotic paramyxoviruses Nipah virus and Hendra virus ⁷ (genus Henipavirus). Among this viral subfamily, the organization and structure of the polymerase appears to be fundamentally conserved. In particular, sequence analysis shows that the final fifty amino acids of the P protein have a strong helical propensity ⁸. For both measles and Sendai viruses, high-resolution structure determination shows this region to be a compact 3-helix bundle ^{9; 10}, and for measles, mumps and Sendai viruses this domain has been demonstrated to be both necessary and sufficient for nucleocapsid binding ^{9; 11; 12}. It has therefore been termed the nucleocapsid-binding domain (NBD) or alternately, the X domain (XD).

While there is limited information about the overall organization of the polymerase, with even its stoichiometry still under investigation ^{13, 14, 15} the experimental evidence linking the C-terminus of the P protein to nucleocapsid-binding is now emphatic. The generally accepted model is that the clustered C-terminal domains of P act as the “feet” of the polymerase, attached by flexible legs ^{10; 16; 17} to the main body of the complex. These feet must repeatedly bind and release the nucleocapsid as the replication machinery moves processively along its template during RNA synthesis

The nucleocapsid is an inherently flexible structure, however image reconstruction, based on cryo-electron micrographs of its tightly wound states, has now revealed its basic architecture ^{18; 19; 20}. The N protein itself is a bipartite molecule, with a structured and globular N-terminal domain, responsible for genomic RNA packaging and formation of the helical nucleocapsid, and a largely unstructured C-terminal tail, varying widely in length and sequence composition, which decorates the nucleocapsid surface.

The site of polymerase attachment for both measles virus and Sendai virus is a short and contiguous sequence within the unstructured tail of the N protein ^{11; 21; 22; 23}, though the amino acid composition of this sequence varies markedly between the two viruses. The interactions between the NBD from the P protein, and the short sequence it recognizes from the N protein have now been characterized in molecular detail for both viruses ^{12; 24}. In each case binding occurs with 1:1 stoichiometry; is relatively weak (dissociation constants in the

μM range); involves the molecular surface created by helices $\alpha 2$ and $\alpha 3$ of the NBD; and is coupled to a disorder-to-order transition within the N tail peptide, which binds to the NBD as an α -helix, transiently creating a four-helix bundle. However in detail, the binding interface is not conserved, being purely hydrophobic in the case of measles virus ²⁴, and dominated by charged amino acids in the case of Sendai virus ¹².

In contrast, for mumps virus the site of NBD attachment is the structured N-terminal domain of the N protein ¹¹. Additionally the mumps P NBD appears to be unstable in solution, lacking an invariant tertiary structure ¹¹. These observations suggest that, while polymerase translocation in mumps virus may still involve coupled binding and folding of protein domains, the roles of the binding partners may be reversed. In this case it appears that the structured region of the N protein may act as the folding “template”, to which the partially structured NBD attaches.

Recently we were successful in crystallizing the mumps P NBD, and have now determined its three-dimensional structure using X-ray diffraction data. The domain exists in a compact and folded state within the crystal. This prompted us to perform further experiments to establish the organization of the NBD in solution. This paper reports the crystal structure of the mumps P NBD, and the results of experiments to probe its solution conformation.

Results

Crystal Structure of the mumps P NBD reveals a conserved fold

The structure of the mumps P NBD was determined from single crystal X-ray diffraction data. The crystals were grown by vapor diffusion at 4 °C, from concentrated salt solutions, and were extremely labile. The multiple wavelength anomalous diffraction method was used to determine phases at 3.0 Å resolution, using data collected from crystals of a Selenomethionine-substituted protein. There are two copies of the NBD within the asymmetric unit of the crystal. The initial structural model was refined and rebuilt using a higher resolution (2.1 Å) native data set. The final crystallographic R-factor was 19.0 %, and the R-free was 24.1% (Table 2).

The mumps P NBD forms a compact 3-helix bundle in the crystal (Fig 1A). The two copies of the domain within the asymmetric unit are essentially equivalent (RMS deviation 0.7 Å for all atoms, 0.2 Å for main chain atoms) and the electron density is well defined throughout (Fig 1B), excepting the loop connecting helices $\alpha 1$ and $\alpha 2$. In terms of the molecular dimensions, and the spatial arrangement of the helices, the domain closely resembles the NBDs from the measles and Sendai virus P proteins (Fig 1C) despite the very low levels of sequence identity ⁸.

That the two copies of the molecule in the asymmetric unit were essentially identical, and the domain exhibited such close structural similarity to the NBDs from Sendai virus and measles virus, strongly suggested that the mumps P NBD has a unique folded state, which has been captured in the crystal. However our earlier observations signaled that this state is not significantly populated under normal solution conditions. While helical secondary structure was previously detected using circular dichroism spectroscopy, application of solution NMR spectroscopy demonstrated that the domain was conformationally heterogeneous ¹¹. Hence we hypothesized that in solution, the mumps P NBD exists in the molten globule state, being neither fully folded nor unfolded

The molten globule-state is usually defined as having a “native-like” secondary structure, a compact overall conformation, yet an ill-defined tertiary structure, lacking the tight and specific packing of internal side-chains characteristic of folded proteins ^{25; 26, 27}. Many folded proteins can transition to the molten globule state under non-native conditions (e.g. in very

acidic solution), and molten globules are frequently postulated as intermediates in protein folding. There are also a few well-characterized examples of proteins that exist as molten globules under normal solution conditions, in the absence of extreme acidification or chemical denaturants (see e.g. ^{28; 29}). We proceeded to investigate if the mumps P NBD was one of these.

The mumps P NBD lacks the thermodynamic characteristics of a completely folded protein

Differential scanning calorimetry (DSC) was used to study temperature-induced conformational changes in the NBD. In this technique, the heat capacity of a protein solution is measured as a function of temperature ^{30; 31}. Following correction for instrumental effects, the DSC profile of a simple protein will usually show a single peak, associated with heat absorption during the transition from the folded to the unfolded state. The area under the peak is related to the total enthalpy change for the transition.

The nucleocapsid-binding domain under study is particularly simple. In the absence of significant stabilizing tertiary interactions it should behave, thermodynamically, as a collection of isolated α -helices, the properties of which are well understood. The unfolding of helices in water is not, in general contrast to globular proteins, an “all-or-none” cooperative transition, and is not adequately described by a two-state model. Additionally the enthalpy associated with the helix to coil transition is rather low ^{32, 33, 34, 35}. As a consequence, DSC profiles of helical peptides exhibit characteristically broad peaks, spanning a large temperature range.

The DSC-derived heat capacity profile of the mumps P NBD is shown in Fig 2A. The unfolding transition for the mumps P NBD is extremely broad and already underway at the lowest temperatures experimentally accessible (7 °C), indicating that the fully structured state must be sparsely populated, even at low temperature. A heat capacity profile of this kind cannot result from a highly cooperative, two-state unfolding transition, as is generally observed for globular proteins. Yet neither is it characteristic of an unstructured polypeptide. As a reference, we plot the profile that would be expected from an unstructured protein with the same amino acid composition, calculated by a group-additivity scheme ³⁶. There is a small vertical offset between the experimental and calculated curves, most obvious at high temperature, where they should converge as the protein completely unfolds. Possible causes are errors in the estimated protein concentration, the approximations made in the processing of the experimental data, and uncertainties in the heat capacities of the constituent groups used in the calculation ³⁶. Notwithstanding this, the experimental profile is not that expected of an unfolded protein. In fact, the heat capacity profile obtained from the mumps P NBD, very closely resembles those arising from isolated helical peptides ^{37, 38, 34}.

In principle DSC data can be further analyzed to extract the change in enthalpy and heat capacity associated with unfolding ^{30; 31}. However the absence of clear pre- and post-transition baselines makes this difficult, and it has not been attempted.

As a control experiment, the measles P NBD was examined using DSC. This domain is stable and highly structured in solution ^{9; 11, 24}. The heat capacity function obtained looks strikingly different (Fig 2B). It is dominated by a relatively sharp peak, centered at 85 °C, with the enthalpy of unfolding evidently much greater than it is for the mumps P NBD. This demonstrates that the behavior of the mumps P NBD is not a consequence of its small size, but is a reflection of its basic structural properties.

An alternate way to study protein thermal stability is to examine secondary-structure content as a function of temperature, using circular dichroism (CD) spectroscopy. At low temperature, CD spectra of the mumps P NBD show the characteristics expected for an α -helical protein; negative bands near 222 and 208 nm, and a positive band at 192 nm ¹¹. In Figure 3 we show

the CD signal at 223 nm, as a function of temperature. Loss of the helical structure begins immediately as the temperature is raised above 0 °C. This observation, together with our earlier estimate¹¹ of the overall helical content of the domain (70% at 2 °C), suggests that the helical structure observed in the crystal does not completely form in solution, even at the freezing point of water. The CD signal increases sigmoidally with temperature as the helical structure gradually dissipates. Complete unfolding occurs at temperatures above 60 °C. The broad and gradual nature of the unfolding transition is again indicative of a largely non-cooperative process.

Thus the spectroscopic and calorimetric results are concordant, and together suggest that, while the mumps P NBD has significant α -helical content at low temperatures, stabilizing tertiary interactions are largely absent, and much of the secondary structure is lost at physiological temperatures.

Complete folding of the Mumps P NBD can be induced by addition of the osmolyte trimethylamine N-oxide

We then examined if it was possible to force folding of the mumps P NBD by addition of the protective osmolyte trimethylamine N-oxide (TMAO). TMAO is known to promote folding of unstable proteins³⁹. The physical basis of this effect is proposed to be the unfavourable interaction between TMAO and the polypeptide backbone, which preferentially raises the free energy of partially unfolded states, shifting the equilibrium toward the folded form of the protein^{40; 41}.

Solution NMR spectroscopy was used to monitor the structural state of the mumps P NBD, collecting 2D ¹H-¹⁵N heteronuclear sequential quantum correlation (HSQC) spectra at several temperatures, in both the presence and absence of 1.56 M TMAO. These experiments allowed direct observation of the resonances associated with the backbone amide groups of the protein.

In the absence of TMAO, the HSQC spectra of the mumps P NBD (Fig 4) shows limited chemical shift dispersion in the proton dimension, large variations in peak intensity, and extreme line broadening, as previously reported¹¹. These are hallmarks of a partially structured protein, exhibiting conformational heterogeneity and complicated dynamic behaviour^{42, 43}. We had earlier eliminated aggregation as a cause of the NMR spectral appearance¹¹. Shifting the temperature from 25 to 5 °C has little effect on the overall appearance of the NMR spectra, although CD spectroscopy measurements show that this does result in an increase in helical secondary structure content (Fig 3)

In contrast, the effect of adding TMAO to the buffer is quite dramatic (Fig 4). At both temperatures, chemical shift dispersion increases markedly, and the resonances devolve into well-dispersed single peaks of fairly uniform intensity. Such spectra are characteristic of a folded domain. Following the addition of TMAO there are ~ 44 discrete peaks in the HSQC spectra (ignoring those resonances clearly arising from side-chain amide groups), accounting for most of the 48 backbone amide groups within the mumps P NBD. Hence it is evident that addition of TMAO, at high concentration, has profoundly shifted the solution equilibria in favour of the folded state.

The mumps P NBD exists in the molten globule state

Since folding of the mumps P NBD could be induced by TMAO, the compactness of the domain was examined as a function of TMAO concentration. If the protein existed in the molten globule state, it should show a small, but measurable, decrease in size on folding. Protein size was monitored using dynamic light scattering. The results are presented in terms of the hydrodynamic radius, the radius of a hypothetical hard sphere diffusing at the same speed as

the protein. This implicitly corrects for the increase in solution viscosity, and corresponding decrease in molecular translational diffusion, that is caused by addition of TMAO.

Addition of TMAO to the mumps P NBD causes a significant contraction in the size of the domain (Fig 5A), which converges to a size-invariant state at TMAO concentrations in excess of 1 M. As deduced from NMR spectroscopy this latter state of the protein is highly structured. The overall change in hydrodynamic radius is ~ 4 Å. We suggest this is consistent with a transition from a molten globule to a fully folded state, with compaction of the domain involving both completion of the helical secondary-structure, as well as condensation of the helices and the formation of stable and invariant tertiary contacts.

As a negative control, we examined the size of the measles P NBD as a function of TMAO concentration. In contrast to the mumps P NBD, the size of the measles P NBD is relatively insensitive to addition of TMAO (Fig 5B). This is the expected result, as the domain is very stable and the initial population of partially folded states is likely to be very small.

Calculation of the hydrodynamic radius from the crystallographic models, using a bead-modelling approach⁴⁴, yields hydrodynamic radii of 15.1 nm (Mumps P NBD) and 15.2 nm (Measles P NBD), somewhat larger than the experimentally determined values. However the hydrodynamic radius depends on the structuring of water at the protein surface^{44, 45}, and this is only approximated in the calculation.

Discussion

The major findings of this paper are that the mumps P NBD has a unique folded state, equivalent in topology and size, to the NBDs from measles and Sendai viruses (Fig 1). However, the folded state has been stabilized by crystallization, and is not significantly populated in solution. The domain has helical secondary structure in solution, but lacks the thermodynamic characteristics of a fully folded protein. The protein heat capacity, profiled as a function of temperature (Fig 2), suggests the helices are behaving largely autonomously. This is supported by the gradual and continual diminution in helical secondary structure that occurs on heating (Fig 3). Solution NMR spectroscopy shows directly that the domain is conformationally heterogeneous, and undergoing complicated dynamic behaviour. Folding of the domain, can however, be forced by addition of the stabilizing cosolute TMAO (Fig 4). Exploiting this finding we demonstrate that the difference in hydrodynamic radius between the native and folded state of the mumps P NBD is around 4 Å, implying the native state is relatively compact (Fig 5). Taken together these observations make a compelling case that the mumps P NBD exists in solution as a molten globule. The folded state, observed in the crystal, must be slightly higher in energy than the conformations that predominate in solution.

Our experimental observations have been made *in vitro*, in dilute solution. In contrast, the viral polymerase functions in the crowded environment of the cytoplasm, where volume exclusion effects will be considerable, and compact states of a protein will be favoured over expanded states^{46; 47}. In this environment the mumps P NBD may well be more structured than direct extrapolation of our thermal unfolding experiments (Figs 2 and 3) would suggest. Indeed, we have shown that addition of the small cosolute, TMAO, dramatically shifts the equilibria governing protein folding, illustrating the general importance of such considerations. This being noted, the NBD from the mumps polymerase is fundamentally unstable, and natively much less structured than the analogous domains from closely related viruses such as measles and Sendai. The tendency of the domain to unfold carries with it an entropic cost for any binding process in which it is involved. Even if the folding equilibria are shifted by crowding effects, or by the complicated chemical environment of the cell, the energetic implications for binding will remain.

Full folding of the mumps P NBD seems to be coupled to crystallization, as the buffer system used to grow the crystals does not, in itself, appreciably stabilize the protein (see Materials and Methods and Supplementary Fig S1). There are many crystal structures of protein complexes, in which one of the molecules is unstable or disordered in solution, yet has been structured by binding to its biological partner. However we are aware of only a limited number of examples in which a protein in the molten globule state has been successfully crystallized by itself^{48, 49}. Presumably this reflects the fundamental improbability of coupling protein folding with crystal nucleation, and having crystal contacts of sufficient density and favourable energy to effect folding. The stabilization energy for typical protein crystals is about 20-40 kJ/mol⁵⁰. This is comparable with the energy stabilizing the folded state of many proteins⁵¹. Because the crystal structure of the mumps P NBD is so similar to its counterparts from measles and Sendai viruses, there is no indication that the process of crystallization has significantly distorted or modified the structure.

The nature of the crystal contacts suggests how the structure may be stabilized in the solid state. The protein is packed quite densely in the orthorhombic crystals, with a Matthews coefficient of 1.88 Å³/Da, corresponding to a solvent content of 35 %. The molecule forms close-packed molecular layers in the XY plane of the crystal (Fig 6), with all helices roughly aligned. This packing arrangement effectively prevents any lateral expansion of the helical bundle, which we posit must occur in solution.

Still to be experimentally determined are the regions of the mumps P NBD that are directly involved in nucleocapsid binding. In Figure 7 we show an alignment of sequences from the members of the Rubulavirus genus. Notable is a strongly conserved motif at the end of helix $\alpha 3$ (consensus sequence IIRSA). Several surface exposed residues (E376, K383) earlier in the helix are also well conserved. In the case of both measles and Sendai viruses, it is the molecular surface created by helices $\alpha 2$ and $\alpha 3$ of the NBD that binds the N protein. However, among the Rubulaviruses, the surface associated residues of helix $\alpha 2$ are poorly conserved. While it is inherently difficult to predict protein-protein interaction sites from sequence conservation⁵², we would suggest that helix $\alpha 3$ is likely to be involved in binding the mumps virus nucleocapsid.

This study confirms that considerable diversity exists in the basic translocation mechanisms of paramyxoviral polymerases; molecular machines that on first inspection appeared to be extremely similar. However in all three members of the Paramyxovirinae studied in detail, attachment of the polymerase to the viral nucleocapsid, via the NBD of the P protein, appears to be coupled with folding of one of the molecules involved. We suggest that the mumps P NBD is likely to bind to a groove or cleft on the structured surface of the mumps virus nucleocapsid, and that the free energy associated with binding will be sufficient to transiently fold the domain. Coupling of protein binding and folding appears to confer modest binding affinity, while retaining the binding specificity required for processive motion of the polymerase⁵³. Another consequence of this arrangement is that the lifetime of the complex is likely to be short (already demonstrated experimentally for measles virus²⁴), which would allow the polymerase to take rapid steps forward.

Finally, as a true molten globule, that can be induced to fold by addition of a stabilizing methylamine cosolute, the nucleocapsid-binding domain from the mumps virus polymerase will also provide an excellent system for study of the protein folding problem.

Materials and Methods

Protein expression and purification

The proteins used in this study, and the nomenclature used throughout the materials and methods section, are summarized in Table 1. All proteins were produced by heterologous expression in *Escherichia coli*. Details of the expression vectors, host bacterial strains, expression conditions, and protein purification procedures have been published previously¹¹. One new expression vector, enabling production of mumps P NBD-Cys+Tyr, was derived from an existing vector for production of mumps P NBD-Cys using a variation of the QuikChange site-directed mutagenesis protocol (Stratagene).

Selenomethionine (SeMet) substituted protein was produced by growing the bacterial host in minimal media; down-regulating methionine biosynthesis; and adding SeMet before induction of heterologous protein expression⁵⁴. Uniform ¹⁵N-labelled protein was prepared by growing the bacterial host in minimal media (for medium composition see⁵⁵) with ¹⁵NH₄Cl as the sole nitrogen source.

Where possible, the concentrations of purified proteins were calculated from UV absorption measurements at 280 nm⁵⁶.

X-ray crystallography

Crystals of mumps P NBD-Cys were grown by the sitting-drop vapour diffusion method. Protein concentration, assessed by band intensity on Coomassie-stained SDS-PAGE, was between 500 and 2000 μ M. Protein solutions were buffered with 12.5 mM MOPS/KOH pH 7.0, 100 mM NaCl and 0.01% (wt/vol) NaN₃. Crystallization conditions were identified with screening experiments based on orthogonal arrays⁵⁷. Fragile, needle-like crystals formed at 4 °C over a period of weeks, in drops equilibrated with 3.6 – 4.6 M Ammonium formate, buffered with 0.2 M Alanine/KOH pH 9.7.

Working at 4 °C, drops were flooded with 5 M Ammonium formate, buffered as before; the crystals suspended in thin fibre loops; and the crystal and surrounding solution vitrified by direct immersion in liquid nitrogen. Crystals were only transiently stable after adding the high-salt solution to the drops. X-ray diffraction data were collected at both the Advanced Light Source (ALS) Beamline 8.2.1 (Berkeley, California) and the Stanford Synchrotron Radiation Laboratory (SSRL) Beamline 9-1 (Stanford, California), maintaining the sample at 100 K in a cold gas stream. Data integration and scaling was performed with the program HKL2000⁵⁸. Morphologically indistinguishable crystals belonged to one of two spacegroups, being either monoclinic (Spacegroup P2₁, unit cell dimensions $a = 37$, $b = 24$, $c = 44$ Å; $\alpha = 90$, $\beta = 95$, $\gamma = 90^\circ$) or orthorhombic (Space Group P2₁2₁2₁, unit cell dimensions $a = 24$, $b = 56$, $c = 60$ Å; $\alpha = \beta = \gamma = 90^\circ$). Geometric considerations and analysis of the self-rotation function⁵⁹ indicated the molecular packing in the two crystal forms was closely related, and in each case there were two protein molecules in the asymmetric unit. The structure was determined using data collected solely from the orthorhombic crystal form. Most crystallographic calculations were carried out using programs from the CCP4 program suite⁶⁰

Using the inverse-beam method, a dual-wavelength MAD dataset was collected at SSRL Beamline 9-1, from a crystal of SeMet-substituted protein (Table 2). The anomalously scattering Se atoms were located using the program SHELXD⁶¹, and phases calculated using the program MLPHARE. An obviously interpretable electron density map, at 3 Å resolution, was improved with solvent flipping/flattening using the program Solomon. This map allowed placement of the six expected alpha helices (three for each domain) and partial assignment of the sequence, using the program COOT⁶². This initial model was then refined against a higher resolution native data set (Table 2) using the program RefMac. After multiple rounds of model

building and refinement, the final crystallographic R / R-free was 19.0 / 24.1 %. Other statistics associated with the model are reported in Table 2. The crystallographic model and X-ray diffraction data used for model refinement have been deposited in the RCSB protein data bank (deposition code 3BBZ)

Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) was carried out using a VP-DSC 2 instrument (MicroCal, Inc). Proteins (mumps P NBD_{-Cys+Tyr}, measles P NBD) were dialyzed into buffer containing 10 mM NaH₂PO₄/Na₂HPO₄ pH 7.0, 100 mM NaCl, 0.01% (wt/vol) NaN₃ and degassed before data collection. Protein concentrations were 0.1 – 3.1 mM. Both the reference scans (having buffer in both cells) and sample scans (having protein in one cell, and buffer in the other) were performed in replicate, over a temperature range of 7 - 90 °C (mumps P NBD_{-Cys+Tyr}) or 27 - 120 °C (measles P NBD), with a scan rate of 1°/min.

From the difference $\Delta C_p(T)$ between the measured heat capacities of the protein solution and the buffer, the partial specific heat capacity of the protein $C_p(T)_{protein}$ was calculated according to 30; 31

$$C_p(T)_{protein} = \frac{\Delta C_p(T)}{m_{protein}(T)} + C_p(T)_{buffer} \frac{V(T)_{protein}}{V(T)_{buffer}}$$

Where $C_p(T)_{buffer}$ is the partial specific heat capacity of the buffer, $V(T)_{buffer}$ is the partial specific volume of the buffer, $V(T)_{protein}$ is the partial specific volume of the protein, and $m_{protein}(T)$ the mass of protein in the calorimeter cell.

The specific heat capacity and specific volume of the buffer were approximated using the values for pure water. The specific heat capacity of water, as a function of temperature, was calculated from a polynomial fit to the data of Stimson⁶³. The specific volume of water, as a function of temperature, was calculated using the expression for water density given by Kell⁶⁴. The specific volume of the protein, as a function of temperature, was calculated from the amino acid composition using a group additivity scheme⁶⁵. The change in mass of protein in the cell, due to differential thermal expansion, was neglected, since the effect is small. The partial molar heat capacity of the protein is obtained from the partial specific heat capacity through multiplying by the molecular weight.

Several control experiments were performed. DSC profiles were obtained for mumps P NBD_{+Tyr}, buffered as above, with the addition of 0.5 mM Tris(2-carboxyethyl)phosphine (TCEP) as a reducing agent. The profiles were essentially identical to those obtained using mumps P NBD_{-Cys+Tyr} (data not shown) verifying that the C356S mutation was not destabilizing. Mumps P NBD_{-Cys+Tyr} was also dialyzed into 200 mM Alanine/KOH pH 9.7, 0.8 M Sodium formate, which was the buffer/salt system used for crystallization. Under these conditions, thermally induced denaturation in the calorimeter was very poorly reversible, hence the results slightly equivocal. However at least qualitatively, the behaviour of the domain was not markedly changed, with the protein exhibiting a broad, low temperature transition, just as it did in neutral pH phosphate buffer (Supplementary Figure S1).

Circular Dichroism (CD) spectroscopy

For CD spectroscopy, mumps P NBD_{-Cys+Tyr} was dialyzed into buffer containing 10 mM NaH₂PO₄/Na₂HPO₄ pH 7.0, 100 mM NaCl, 0.01% (wt/vol) NaN₃. Measurements were made using a JASCO J-810 spectrophotometer, with the protein (1.8 μM) in a 1cm optical cell.

Thermal unfolding of the protein was monitored by following the CD signal at 223 nm as a function of temperature (0 – 74 °C), as previously described⁶⁶

Solution NMR spectroscopy

For solution NMR spectroscopy, uniform ¹⁵N-labeled mumps P NBD-_{Cys} was transferred into a buffer containing 10 mM NaH₂PO₄/Na₂HPO₄ pH 5.7, 50mM NaCl, 0.5 mM NaN₃. Solid trimethylamine N-oxide (TMAO) was dissolved in the same buffer, to a final concentration of 3.0 M. Samples for spectroscopy were prepared by diluting the protein solution with an identical volume of either the buffer, or the buffer + 3M TMAO. 7% (v/v) ²H₂O was added to both samples before NMR data acquisition.

2D ¹H-¹⁵N heteronuclear quantum sequential correlation spectra were recorded on the samples at 5 – 25 °C using a Bruker 600 MHz spectrometer, equipped with a triple-resonance pulsed-field gradient cryoprobe. NMR data were analyzed using the program NMRPipe⁶⁷.

Dynamic Light Scattering

For Dynamic light scattering (DLS), mumps P NBD-_{Cys+Tyr} and measles P NBD were dialyzed into a buffer containing 10 mM NaH₂PO₄/Na₂HPO₄ pH 7.0, 100 mM NaCl, 0.5 mM NaN₃. Samples for light scattering were prepared by diluting stock protein solutions with fixed volumes of buffer containing varying concentrations of TMAO. All DLS measurements were carried out at 18 °C. At the protein concentrations studied (< 600 μM), the data were not significantly concentration dependent, so results were not extrapolated to infinite protein dilution.

DLS measurements were made using a DynaPro M540 instrument (Protein Solutions, Inc.) at a wavelength of 836 nm. The normalized (baseline-corrected) intensity autocorrelation functions $G(\tau)$ produced by the instrument were fitted directly to the equation expected for scattering from monodisperse solutions of non-interacting molecules⁶⁸.

$$G(\tau)=1+\alpha e^{-2q^2 D_T \tau}$$

where α is an instrument constant, D_T is the translational diffusion coefficient, and q is the length of the scattering vector, given by

$$q=\frac{4\pi n}{\lambda}\sin(\theta/2)$$

where n is the refractive index of the buffer, λ the wavelength of the incident light, and θ the scattering angle. The refractive index of solutions was measured at 589 nm using an Abbe-3L refractometer (Bausch and Lomb). The dispersion in the refractive index between 589 and 836 nm was neglected. Fitting of the single exponential decay model to the experimental data was achieved using non-linear least squares. Model residuals were random, giving no indication that a more complicated model was needed to fit the data.

The measured diffusion coefficients were used to calculate the hydrodynamic radius (R_H) of the particle using the Stokes-Einstein relationship.

$$R_H = \frac{k_B T}{6\pi\eta D_T}$$

Where k_B is the Boltzmann constant, and η the dynamic viscosity of the buffer. Solution viscosity was measured using a Cannon-Ubbelohde viscometer (Cannon Instrument corporation). Solution density was measured on a conventional mass balance.

Hydrodynamic radii were calculated from crystallographic models using the bead-modelling approach of De La Torre *et al*⁴⁴, and a common atomic radius of 3.2 Å.

Structure analysis and figure preparation

Structures were optimally aligned using the SSM algorithm⁶⁹. The programs PyMol (<http://pymol.sourceforge.net/>) and Grace (<http://plasma-gate.weizmann.ac.il/Grace/>) were used in the preparation of the figures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
The structure of the mumps P nucleocapsid-binding domain. (A) Schematic ribbon diagram showing the arrangement of the α -helices. (B) Representative electron density within the crystal. The map is a $2m|F_o|-D|F_c|$ (SigmaA-weighted) Fourier synthesis. (C) Aligned C α plots of the mumps, measles, and Sendai virus NBDs.

Figure 2.

Protein partial molar heat capacity as a function of temperature. (A) Mumps P NBD. (B) Measles P NBD. The partial molar heat capacity, derived from differential scanning calorimetry data, is indicated with filled circles. Results from three consecutive scans using the same sample are shown. The thermally induced denaturation of the mumps P NBD is nearly reversible, with repeat scanning resulting in a slight attenuation of the peak associated with the unfolding transition. Thermal unfolding of the measles P NBD exhibits limited reversibility, most probably because of the high temperatures required to fully denature the protein, which facilitate chemical degradation. Protein concentrations for the scans were 3100 μ M (mumps P NBD) and 220 μ M (measles P NBD). The solid lines represent the theoretical heat capacity of the completely unfolded state, calculated using a group-additivity scheme³⁶.

Figure 3. Circular dichroism at 223 nm, as a function of temperature, for the mumps P NBD.

Figure 4.
2D ^1H - ^{15}N Heteronuclear quantum sequential correlation (HSQC) spectra of the mumps P NBD, in the presence or absence of 1.56M TMAO.

Figure 5.

Hydrodynamic radius as a function of TMAO concentration. (A) Mumps P NBD (B) Measles P NBD. Dynamic light scattering data were used to estimate translational diffusion coefficients, from which hydrodynamic radii were calculated using the Stokes-Einstein relationship (see Materials and Methods). The mean hydrodynamic radius, resulting from several hundred individual estimates, is displayed. The error bars show the 95% confidence interval for the mean. Measurements were made at fixed protein concentrations of 390 μ M (mumps P NBD) and 580 μ M (measles P NBD), and a temperature of 18 $^{\circ}$ C.

Figure 6.

Close packed layers of the mumps P NBD formed within the crystal. (A) Schematic ribbon diagram showing the packing arrangement of molecules in the XY plane of the crystal. The two molecules within the asymmetric unit are shown in light blue. Symmetry-equivalent molecules are shown in grey. For clarity only a single molecular layer is shown. As depicted, the non-crystallographic two-fold rotation axis lies parallel to the crystallographic Z-axis. (B) A molecular (Connolly) surface representation of the region shown in (A) calculated using the program PyMol.

Figure 7. Sequence conservation within the NBD, across the Rubulavirus genus. (A) Alignment of representative sequences from each virus in the genus (B) A Sequence Logo⁷⁰ graphical representation of the multiple sequence alignment, numbered according to the mumps P sequence. The positions of the α -helices observed in the mumps P NBD structure are indicated schematically. Secondary structure assignments were made using the method of Kabsch and Sander⁷¹

Table 1

Recombinant proteins used in this study

Short name	Molecular details	Rationale for mutations
Mumps P NBD _{-Cys}	Mumps P (Jeryl Lynn vaccine strain) amino acids 343-391, with mutation C356S	Mutation C356S limits chemical reactivity of the domain.
Mumps P NBD _{+Tyr}	Mumps P (Jeryl Lynn vaccine strain) amino acids 343-391, and a C-terminal tyrosine	C-terminal tyrosine allows protein quantitation through UV absorbance measurements.
Mumps P NBD _{-Cys+Tyr}	Twin variant, details as above	As above
Measles P NBD	Measles P (Moraten vaccine strain) amino acids 457-507, with mutation P458G	Mutation P458G facilitates proteolytic cleavage of the progenitor GST fusion protein.

Table 2
X-ray diffraction data and model refinement statistics

X-ray diffraction data			
Data set	SeMet		Native
Cryoprotectant	0.2 M Alanine/KOH pH 9.7, 5M Ammonium Formate		0.2 M Alanine/KOH pH 9.7, 5M Ammonium Formate, 1M Sodium Bromide
Space group	P2 ₁ 2 ₁ 2 ₁		P2 ₁ 2 ₁ 2 ₁
Unit cell lengths (Å)	a = 23.7 b = 56.3 c = 60.7		a = 24.0 b = 55.5 c = 60.0
Unit cell angles (°)	$\alpha = \beta = \gamma = 90$		$\alpha = \beta = \gamma = 90$
X-ray source	SSRL Beamline 9-1		ALS Beamline 8.2.1
X-ray wavelength (Å)	$\lambda_1 = 0.954$	$\lambda_2 = 0.979$	0.920
Sample Temperature (K)	100	100	100
Data resolution limits (Å)	41 - 3.00 (3.11 - 3.10) ^a	41 - 3.00 (3.11 - 3.10) ^a	41 - 2.10 (2.18 - 2.10) ^a
Total number of observations	1845 (169) ^a	1848 (170) ^a	4915 (484) ^a
Mean Redundancy	10.1 (10.7) ^a	12.9 (13.8) ^a	8.4 (8.7) ^a
Completeness (%)	99.8 (100.0) ^a	99.9 (100.0) ^a	97.2 (98.6) ^a
R _{measure} ^b	13.7 (33.3) ^a	17.0 (40.2) ^a	14.0 (34.3) ^a
Mean I / σ I	5.7 (2.4) ^a	5.5 (2.2) ^a	5.8 (2.4) ^a
Model refinement			
Number of protein molecules in the asymmetric unit	-		2
R _{work} / R _{free} (%)	-		19.0 / 24.1
Total number of atoms	-		823
Number of water molecules	-		52
Other ligands	-		6 Formate ions, 2 Bromide ions
Mean isotropic B-factor, all atoms (Å ²):	-		11
Bulk Solvent model	-		Simple mask
RMSD from ideal geometry : Bond lengths (Å) / Bond angles (°)	-		0.020 / 1.6
Residues in most allowed regions of Ramachandran plot (%)	-		97

^aNumbers in parentheses are for the highest resolution shell

^bMultiplicity-corrected measure of agreement according to Diedrichs and Karplus⁷²

^cCalculated from a randomly selected 5% of observations omitted from all model refinement