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PII: S0022-2836(09)00736-0
DOI: doi:10.1016/j.jmb.2009.06.029
Reference: YJMBI 61521

To appear in: Journal of Molecular Biology

Received date: 7 April 2009
Revised date: 8 June 2009
Accepted date: 10 June 2009


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NMR structure of the N-terminal domain of capsid protein from the Mason-Pfizer monkey virus

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Keywords
M-PMV, betaretroviruses, capsid protein, NMR structure; internal dynamics; salt-bridge.

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Summary
The high resolution structure of the N-terminal domain (NTD) of the retroviral capsid protein (CA) of Mason-Pfizer monkey virus (M-PMV), a member of Betaretroviruses family, has been determined by NMR. The M-PMV NTD CA structure is similar to the other retroviral capsid structures and is characterized by a six-α-helical bundle and an N-terminal β-hairpin, stabilized by an interaction of highly conserved residues, Pro1 and Asp57. Since the role of the β-hairpin has been shown to be critical for formation of infectious viral core, we also investigated the functional role of M-PMV β-hairpin in two mutants, i.e., ΔP1NTDCA, and D57ANTDCA, where the salt bridge stabilizing the wild-type structure was disrupted. NMR data obtained for these mutants were compared to that for the wild-type. The main structural changes were observed within the β-hairpin structure, within helices 2, 3 and 5, and in the loop connecting helices 2-3. This observation is supported by biochemical data showing a different cleavage pattern of the wild-type and the mutated capsid – nucleocapsid fusion protein (CANC) by M-PMV protease. Despite these structural changes, the mutants with the disrupted salt-bridge are still able to assemble into immature, spherical particles. This confirms that the mutual interaction and topology within the β-hairpin and helix 3 might correlate with the changes of interaction between immature and mature lattice.

Introduction
Mason-Pfizer monkey virus (M-PMV) belongs to a family of Betaretroviruses that is characteristic by an assembly of immature viral particles in the cytoplasm prior to their transport to the plasma membrane and budding from the infected cells. Similar to other retroviruses, M-PMV is an enveloped virus with two copies of genomic RNA packed within a mature core. In contrast to the C-type retroviruses, for which Human immunodeficiency virus
(HIV-1) may serve as a prototype, assembly of the D-type Mason-Pfizer monkey virus is driven by polymerization of Gag polyproteins within the cytoplasm of the infected cells. Resulting immature spherical particles are transported and bud through the plasma membrane. A viral protease cleaves the Gag polyprotein precursors, enabling formation of infectious virions outside of the infected cell.

The mature, cylindrical core of M-PMV contains a major structural protein, the capsid protein CA (p27). This protein is initially synthesized as a part of the Gag polyprotein precursor, from which it is released by a proteolytic cleavage together with matrix protein MA (p10), phosphoprotein (pp24), protein p12, nucleocapsid protein NC (p14), and protein p4. Three of these structural proteins, namely MA, CA, and NC, are common to all retroviruses. After the proteolytic cleavage, the proteins rearrange to form the mature, infectious virions. The matrix protein remains associated with the viral membrane in mature virions. Recently, it has been shown that the M-PMV matrix protein interacts with Tctex-1 and through the dynein motor machinery mediates the transport to the cytoplasmic assembly site. The capsid protein forms the protein core that surrounds the nucleocapsid-RNA complex together with the viral enzymes, reverse transcriptase (RT) and integrase (IN). Although assembly of the immature retrovirus particles occurs at different places, their shape is spherical regardless of the virus type. In contrast, the shape of a mature retroviral core varies significantly among different genera: cylindrical shape is observed in Betaretroviridae (M-PMV), conical in Lentiviridae (HIV-1), and spherical in Alpharetroviridae, Gamma-retroviridae, Deltaretroviridae, and in Spumaretroviridae. Although the retroviral capsid proteins do not exhibit significant sequence similarity, the tertiary structures of all three-dimensional structures solved to date are remarkably similar. Retroviral CA proteins are comprised of two structural domains, the N-terminal assembly domain (NTD) and the C-terminal dimerization domain (CTD), connected by short flexible linkers. Mutational analyses have demonstrated that
changes in the NTD affect especially the mature core assembly, while mutations in the CTD influence the formation of immature particles\textsuperscript{13-15}.

All structures of the N-terminal domains of retroviral capsid proteins of HIV-1 \textsuperscript{5, 6, 12}, Rous sarcoma virus (RSV) \textsuperscript{2, 9}, Human T-cell leukemia virus (HTLV) \textsuperscript{3, 8}, Equine infectious anemia virus (EIAV) \textsuperscript{7}, N-tropic murine leukemia virus (N-MLV) \textsuperscript{16}, B-tropic murine leukemia virus (B-MLV) \textsuperscript{10} and Jaagsiekte sheep retrovirus (JSRV) \textsuperscript{11} solved to date are composed of six to seven α-helices with almost identical topology. In addition, the N-terminal regions form well defined β-hairpin structures with exception of the NTD CA protein structures of RSV and EIAV solved by NMR and X-ray, respectively, where the β-hairpin is missing. The β-hairpins are formed only upon Gag polyprotein processing, during which the N-terminal proline is released, and the structure is then stabilized by formation of a salt bridge between the released proline and a highly conserved aspartate D51 in HIV-1 \textsuperscript{6}, D52 in RSV \textsuperscript{9}, and D54 in HTLV-1 \textsuperscript{3}, D54 in N-MLV \textsuperscript{16}. Point mutations throughout this region block the proteolytic processing of HIV-1 \textsuperscript{14}, Moloney murine leukemia virus (MoMLV) \textsuperscript{17}, and M-PMV \textsuperscript{18}, and affect the virus core assembly and infectivity of HIV-1 \textsuperscript{14, 19-21} and M-PMV \textsuperscript{18}. Although the mutation data suggests that the β-hairpin formation is important for viral maturation and proper core assembly, its exact role remains unclear. Von Schwedler et al. \textsuperscript{14} suggested that the proteolytic refolding and formation of the β-hairpin creates a new CA-CA interface in the mature capsid core. This model is supported by the data obtained also for HIV-1 \textsuperscript{14, 15, 20} and M-PMV \textsuperscript{22}, which indicates that the formation of the β-hairpin structure can shift the assembly from immature, spherical particles, when the β-hairpin is absent, to mature, tubular cores, when the β-hairpin is present. However, structuring of the β-hairpin is not crucial for the assembly of immature particles since the deletion of the entire β-hairpin (Δ20CANC) does not prevent virus assembly into the spherical particles (Rumllová, unpublished data). In their X-ray crystal structure, Mortuza and colleagues first showed a hexameric arrangement of B-
MLV NTD CA and suggested that six β-hairpins might form an extended interaction network within the mature CA hexamer. Recently, using results of electron cryomicroscopy and image analysis of hexameric arrays of full-length HIV-1 CA, Ganser-Pornillos and colleagues proposed that the β-hairpin may stabilize assembly-competent conformations of important residues in helix 1 and 2 of HIV-1 NTD CA and/or that β-hairpin formation disrupts interactions that stabilize the immature lattice.

Here we present the NMR structure of NTD CA of a Betaretroviridae family member, M-PMV, with a special focus on a missing piece in the mosaic of available information, namely on comparison of the wild-type with the constructs where residues critical for the β-hairpin formation were mutated. The structure of the wild-type NTD CA comprising six helices and an N-terminal β-hairpin, stabilized by an interaction of Pro1 and Asp57, is very similar to the other retroviral NTD CA structures. We also show the NMR data of two mutants, ΔP1 NTD CA and D57A NTD CA. Both these mutations dramatically influenced the structure within the region of β-hairpin and helices α3 and α5, which then became susceptible to cleavage by M-PMV protease. The effect of mutations was investigated also in the CANC fusion protein that was previously shown to be assembly competent in its wild-type form. Despite the structural changes of the NTD CA, both mutations introduced into the CANC fusion protein resulted in assembling spherical immature particles, suggesting that the exact wild-type alike positions of helices α1-α3 and helix α5 of M-PMV CA is not crucial for their assembly.

Results

Solution structure of M-PMV NTD CA

The three dimensional structure of the M-PMV NTD CA was calculated based on NOE distances, backbone φ and ψ dihedral angles, hydrogen bonds, and residual dipolar
couplings (RDCs) as described in Materials and Methods. Table 1 shows summary of the restraints used for the structure calculation. The superposition of ten structures with the lowest energy is shown in Figure 1 and their structure quality statistics is summarized in Table 1 (for additional structural statistics see Table S1 in Appendix A). No distance and dihedral angle restraints were violated among 50 structures with the lowest energy deposited in PDB (PDB code 2kgf). The Q-factor for RDCs was 0.225. The axial component (normalized to the H-N bond) and rhombicity of the RDC tensor optimized during the structure calculation were (-8.73±0.19) Hz and 0.45±0.04, respectively. These values are close to those determined from the RDC distribution.

WHATIF Z-scores were superior to those calculated for NMR structures of related retroviral capsid proteins (PDB codes 1G03, 1GW), with the exception of RSV (PDB code 1D1D, Z-score of -2.7). Backbone torsion angles of five residues occasionally occurred in the disallowed regions of the Ramachandran plot: Asp97 in four structures, Thr102 in two structures, Arg14, Asp117, and Val137 in one structure. With the exception of Arg14, these residues are located in the loop or terminal regions (see below). The negative WHATIF Z-scores in Table 1 reflect somewhat lower quality of the refined structures with respect to the reliable X-ray structures in the PDB database.

Figure 2 shows a ribbon diagram of the structure with the lowest energy. The M-PMV NTD CA structure is characterized by a five-membered α-helical bundle with helical axes in an antiparallel arrangement and by an N-terminal β-hairpin. Helices of the bundle are formed by residues 21-33 (helix α1), 39-48 (helix α2), 55-65 (helix α3), 69-92 (helix α4), and 118-132 (helix α6). Last two helices of the bundle are connected through a twenty-four residue
chain including a partially ordered loop (residues 93-109) and a short α-helix (helix α5, residues 110-114). Among the retroviral capsid proteins, this region is the least conserved and significantly varies in length. The N-terminal β-hairpin, which strands are formed by residues 2-7 and 11-16, is in a parallel arrangement to the axes of the α-helices. The orientation of the β-hairpin with respect to the α-helical core is determined by 14 NOEs defining distances between residues 2-52, 4-52, 2-53, 16-110, and 2-114 and positioning Pro1 near to Asp57. The close proximity of Pro1 and Asp57 indicates the salt bridge formation between positively charged Pro1 and negatively charged Asp57.

**Backbone $^{15}$N dynamics of M-PMV NTD CA**

To assess dynamic properties of M-PMV NTD CA on the picosecond/nanosecond timescale, the quantitative measurement of the longitudinal relaxation time, $T_1$, the transverse relaxation time, $T_2$, and the steady-state $^{1}H-^{15}$N NOE enhancement (ssNOE) was performed. Experimental values of $T_1$, $T_2$, and $^{1}H-^{15}$N NOE were obtained for 111 out of 135 protonated backbone $^{15}$N nuclei. Plots of $T_1$, $T_2$, and $^{1}H-^{15}$N NOE against residue number are presented in Figure 3.

![Figure 3](image.png)

Relaxation times and steady state NOE enhancement are sensitive probes of backbone N-H vector fluctuations. The ssNOE values lower than 0.65 indicate significant internal motion. The average ssNOE of α-helical bundle residues (residues 19-132) was 0.78 ± 0.08. Within the α-helical core, there were 3 residues with the ssNOE value lower than 0.65: Val48 (0.56), Asn51 (0.26), and Gln90 (0.56). The average ssNOE value of the C-terminus was 0.43 (residues 133-140) indicating increased mobility of the unstructured C-terminal tail. The ssNOE values of residues of the β-hairpin decreased from the value 0.84 of the Val2 to 0.57 of the Ala12, and than increased again to 0.82 of the His15. It is in a good agreement with a
model involving Pro1 bound to the Asp57 and β-hairpin with increased flexibility in the turn region.

Relaxation data were interpreted in terms of overall tumbling, described by an asymmetric diffusion tensor, and of internal motions, characterized by generalized order parameter $S^2$, internal correlation time, relaxation exchange rate $R_{ex}$, order parameter for the fast motion, and the internal correlation time for the slow motion, and of overall tumbling, described by an asymmetric diffusion tensor. Its determined principal elements were $11.176 \mu s^{-1}$, $13.160 \mu s^{-1}$, and $16.088 \mu s^{-1}$. Residues exhibiting slow conformational exchange, including Pro1, loop between the β-hairpin and helix α1 (residues 16-20), partially ordered loop between helix α4 and helix α5 (residues 103-109 and 111), residues of helix α6 (119, 120, 122, 125), and residues 36, 57, 60, 70-72, 79, 91, 93, 94, were identified by analysis of spectral densities. Average value of the generalized order parameter $S^2$ was equal to $0.91 \pm 0.13$. Lower $S^2$ values indicating higher flexibility were determined in helix α5, in loops and in the C-terminal region, reflecting increased flexibility on the ns/ps timescale. All parameters fitted in the analysis of the internal dynamics can be found in the Appendix A (Figure S7).

Values of the harmonic mean rotation correlation time predicted by the HydroNMR calculation were compared with the experimental values, derived from the relaxation measurements. In an ensemble of 50 refined structures with the lowest energy, the predicted values were close to the experimental correlation time for the structures with the least compact shape (Figure 1). An alternative explanation of the difference between predicted and experimental rotation correlation time might be partial multimerization of the protein. However, no evidence of oligomerization was observed in dilution experiments and measurements of the $^1$H-$^{15}$N HSQC spectra using 1.0mM and 0.05mM samples (Figure S1, Appendix A)
Salt bridge point mutations

As shown recently, destabilization of β-hairpin in M-PMV CA, by mutation either of P1 or D57 (equivalent to D51 in HIV-1), has a dramatic effect on formation of the mature core of the released virus and completely blocks its infectivity\(^{18}\).

To elucidate whether the mutations altered the structure of the CA NTD, the combined chemical shift perturbations (CCSP) of backbone amide groups were monitored in NMR spectra of a CA NTD mutant, with deletion of N-terminal proline (ΔP1 CA NTD), and of CA NTD, containing D57A replacement (D57A CA NTD) (Figure S6, Appendix A). The $^1$H-$^{15}$N HSQC spectra of both mutants exhibited a large dispersion of chemical shifts in the proton dimension, indicating that both mutants are well ordered (Figure S2, Appendix A). Assignments of the $^1$HN, $^{15}$N, $^{13}$C\(^{\alpha}\), and $^{13}$C\(^{\beta}\) nuclei were obtained from HNCACB and CBCA(CO)NH spectra. Out of 135 possible $^1$HN and $^{15}$N resonances, 127 and 68 were obtained for ΔP1 CA NTD and D57A CA NTD, respectively. In contrast to the wild-type CA NTD (WT CA NTD), His15 and Phe19 of the ΔP1 mutant could not be assigned, while Gly69, not assigned in the wild-type, was identified unambiguously. The Pro1 deletion resulted in substantial peak displacement with CCSP higher than 0.1ppm for residues Trp52, Thr54, Leu61, Met114, and Gln115 located in the loop between helices 2 and 3, in the helix 3, and in the helix 5. Residues 18, 48, 56, 57, and 59 positioned within the loops between β-hairpin and helix 1 and helixes 2 and 3, display CCSP between 0.06ppm and 0.1ppm. The smallest peak displacement is exhibited by residues 70-110 (on average 0.01ppm) located in the helix 4 and long partially ordered loop.

Differences between the D57A mutant and the WT CA NTD $^1$H-$^{15}$N HSQC spectra were more dramatic as 77 residues were not assigned: 2-27, 40, 45-54, 56-61, 68, 69, 90-96, 99-110.
The CCSP of the D57A mutant is higher than 0.1 ppm for 10 residues (29, 32, 62, 65, 112-115, 130, 132) whereas for 9 residues (30, 44, 63, 71, 74, 80, 82, 109, 129) the CCSP is in the 0.06 ppm to 0.1 ppm range.

A ribbon representation of the WT NTD CA color coded according to CCSP induced by ΔP1 and D57A mutation is shown in Figures 4A and 4B for ΔP1 NTD CA and D57A CA NTD, respectively. Residues without backbone amide group assignment in WT CA NTD and proline residues are in grey color, while residues which could be assigned in the wild-type but not a in particular mutant are colored by magenta. Residues with CCSP>0.1 are shown in red and residues with CCSP<0.1 are colored using blue-green-red coding.

Figure 4

To assess ordering at the secondary structure level of the studied variants of NTD CA, far-UV CD spectra were measured. All proteins showed one maximum at 195 nm and double minima at 208 and 222 nm, which are typical of α-helical structures. The far-UV CD spectra of measured proteins slightly differed in the Θ_{222}/Θ_{208} ratio and also in the intensity suggesting that the inserted mutations may have an effect on specific packing of amino acids in the secondary structure of mutant variants, especially on an overall number of amino acids contributing to the α-helical and β-sheet content (Figure S3, Appendix A).

Secondary structures of CA NTD mutants as well as WT CA NTD determined from the CD spectra are listed in Table 2. The quantitative evaluation of a content of helical and β-sheet parts of the protein structure revealed a small secondary structure variation among the measured proteins. Proportion of the β-sheet is lower in the D57A CA NTD (11.0 %) and ΔP1 CA NTD (12.4%) in comparison to the WT CA NTD (14.7%), while somewhat higher helical content was estimated for both mutants. Despite of the large CCSP values indicating substantial variations of tertiary structure introduced by the mutations, the assigned chemical shifts did not reveal any significant changes on the secondary structure level (see Figure S8 in
Appendix A). Although the predicted content of helices from the chemical shifts is higher than the estimation obtained by the analysis of CD spectra, both results showed that the mutations did not disrupt the secondary structure of CA NTD.

The NMR data, showing the dramatic change of the overall structure of D57A CA NTD, correlates with our finding that this mutation caused an improper processing of the Gag polyprotein during virus maturation. This suggests that the D57A mutation might influence not only the CA NTD structure itself but also folding of adjacent regions within the Gag polyprotein. While the function of the N-terminal proline (P1) as a “shape determinant” whose masking by a short extension redirects the assembly from spherical to tubular structure is well known for HIV-1, M-PMV and RSV, the role of its interacting partner, i.e., aspartate residue was not studied in detail. To examine whether the D57 residue can affect the shape of the M-PMV virus like particles (VLPs) similarly to ΔP1, D57A mutation was introduced into M-PMV CANC fusion protein. Bacterial expression/assembly system and *in vitro* assembly assay were employed to study the effect of the introduced mutation. *E. coli* BL21(DE3) cells were transformed with these constructs and the thin sections of the cells expressing the ΔP1CANC or CANCD57A proteins were analyzed using electron microscopy (EM). Structures closely resembling to immature (spherical) particles for both ΔP1CANC and CANCD57A were observed within the bacterial intracytoplasmic inclusions (not shown). To analyze the ability to assemble *in vitro* into organized structures, both ΔP1CANC and CANCD57A proteins were purified as described in Material and Methods, mixed with nucleic acid, and dialyzed against the assembly buffer. The negatively stained protein samples were investigated by EM. As shown in Figure 5, the CANCD57A forms spherical particles similar to those assembled from ΔP1CANC. This result indicates that blocking the salt bridge formation by the D57A replacement produced particles with the same phenotype as particles produced from the constructs in which the N-terminal proline was removed. A mutation of
either of these amino acids switches both the bacterial and the \textit{in vitro} assembly of CANC fusion protein from tubular, mature like, to spherical, immature like particles.

Figure 5

We showed previously that M-PMV CANC protein is cleaved by M-PMV protease into the CA and NC proteins, however, CA is further cleaved within the major homology region (MHR) to yield 17 kDa cleavage product \cite{29}. To analyze whether the mutations which induce structural changes of CA affect the processing pattern, \textit{in vitro} cleavage of purified wild-type CANC, ΔP1CANC and D57ACANC proteins with recombinant M-PMV protease was carried out (Figure 6).

Figure 6

Products corresponding to a specific cleavage of the wild-type CANC between CA and NC as well as additional protein representing the previously reported 17 kDa cleavage product were observed at Western blots in all three samples (Figure 6, panel B). Unlike to the wild-type CANC, another cleavage product of molecular weight 20kDa that was recognized by anti-CA antibody was detected in both mutants, ΔP1CANC and D57ACANC. The products of the CANC digestion were characterized by an N-terminal sequencing analysis. The 17kDa cleavage products derived from all three fusion proteins, shared the N-terminal sequence of CA, i.e., PVTET. Based on the molecular mass determined, this cleavage product was attributed to the 1-158 fragment of CA, reported earlier \cite{29}. Edman degradation of the 20kDa product however revealed the N-terminal sequence IVESV, corresponding to the 44-226 fragment of CA. The new cleavage site PYTLA84**44IVESV resides within the helix 2 of CA NTD. The same cleavage pattern was also identified in the M-PMV released virus with P1A and D57A mutations \cite{18}. These results clearly demonstrate that the helix 2 of ΔP1CANC and D57ACANC mutants became more susceptible to the protease cleavage most likely due to different packing of the helical bundle.
Discussion

The superfamily of N-terminal domains of retroviral capsid proteins is an example of highly conserved structures despite their low level of sequence homology (Figure S4, Appendix A). Structure of the five-helical bundle is very similar in all retroviral CA NTDs (Figure 7). The most significant differences occur in the cyclophilin binding loop, connecting helix 4 to the C-terminal alpha-helix, and in the N-terminal β-hairpin.

Figure 7

The region spanning residues 85-93 of HIV-1 CA NTD is termed cyclophilin binding loop according to its ability to bind cyclophilin A\(^5\). The binding of CypA to this domain of HIV-1 CA helps the virus to overcome the restriction by cellular protein TRIM5α. This mechanism seems to be specific for HIV-1 and the CypA like binding domain varies among the CA NTD structures in length and conformation. Therefore, the variations in this region of the structural models presented in Figure 7 are likely to be related to real differences in the physical behaviour of the molecules. M-PMV is not restricted by TRIM5α in its natural host cells and it has been shown that M-PMV does not bind CypA\(^30\). However, an interaction of M-PMV and TRIM5α in New World monkey cells has been reported\(^31\), proposing binding of TRIM5α to the capsid protein. Compared to the other CA NTD structures, M-PMV CA NTD has a significantly shorter region reminiscent of cyclophilin binding loop with a single regular helical motif, while the corresponding regions of CA NTD structures of most of the other retroviruses contain two short helices. However, analysis of the measured chemical shifts and RDCs indicated signs of helical conformation for residues 105-107 and 103-105, respectively. As the NMR relaxation data revealed an increased flexibility of residues 103-112, it is possible, that the experimental restraints measured for this region represent an average value for a broader ensemble of structures present at room temperature. Such restraints cannot
confine a single physically meaningful conformation in the refinement. It may also explain the lower backbone normality WHATIF score of associated residues (Figure S5, Appendix A).

The N-terminal β-hairpin has different orientations in CA NTD structures in various retroviruses shown in Figure 7. It should be noted that the position of the β-hairpin varies significantly in the ensemble of 50 lowest-energy structures (Figure 1). The higher flexibility detected in the connection between the β-hairpin and helix 1 and in the adjacent loop between helices 2 and 3 suggests that the observed uncertainty in the hairpin orientation is not mere consequence of missing experimental restraints but reflects real dynamics of the molecule. On the other hand, hydrodynamic calculations indicate that the refined ensemble of structures does not completely describe the actual range of conformations probed by the relaxation measurements.

The position of the β-hairpin relative to the α-helical core is fixed by a salt bridge between the N-terminal Pro1 and Asp57 (see Figure 8 for detail) and by the electrostatic interactions of the β-hairpin residues (14,16) to the short helix α5 (111, 117). Similar interactions between the highly conserved Pro1 and Asp at the beginning of helix three were found in structures of HIV-1, RSV, HTLV, B-MLV, and JSRV.

Figure 8

It was suggested that processing of the Gag polyprotein triggers the β-hairpin folding that is induced by a formation of a salt bridge. This structural rearrangement is believed to be a morphological switch to formation of the mature capsid. To investigate the destabilization of the β-hairpin upon the salt bridge disruption, the effect of P1 deletion and D57A replacement on the backbone chemical shifts of amide groups of M-PMV NTD CA was analyzed. Changes induced by the P1 deletion were localized predominantly in the vicinity of residues involved in the β–hairpin formation and stabilization, but the loops connecting the
helices 2-3, and the helices α3 and α5 were also affected. The changes in the D57A mutant were more dramatic as a large portion of resonances except of the helix α4 was significantly shifted. Many of the changes were scattered through the helices α1, α2, α3, α5, and α6 (Figure 4B). We can speculate that mutation of Asp57 destabilizes not only the β-hairpin but also inter-helical interactions of helices α2, α3, and α5.

As mentioned above the structural changes both in ΔP1 and D57A mutants induced the ability of purified CANC protein to assemble into spherical particles in vitro. This is consistent with the concept that β-hairpin is required for the formation of mature core but it prevents assembly of spherical particles. In contrast, the capability to form spheres remains unchanged in both mutants, similarly to the situation where the N-terminus of CA is covalently attached to the upstream sequence of the Gag polyproteins and thus incapable to form β-hairpin 18. These results suggest that the N-terminal part of CA protein is an important shape determinant and its correct folding is required for the assembly of immature particles. Interestingly, deletion of the N-terminal 20 amino acids of M-PMV CANC did not abrogate the in vitro assembly of spherical particles (unpublished results).

Both mutations ΔP1 and D57A influenced the virus maturation by improper processing of structural Gag polyproteins. Besides the expected products, an internal cleavage site, i.e., PYTLA13-44IVESV, within the helix 2 of CA, yielding a 20 kDa protein, was detected after in vitro proteolytic cleavage with M-PMV protease in both ΔP1CANC and D57ACANC mutants. Therefore it is highly probable that mutations ΔP1 and D57A that destabilize the β-hairpin induce relaxation of the α-helical bundle that opens an access to this cryptic cleavage site for promiscuous retroviral protease.

The effect of the salt bridge disruption by the mutation of Asp in the helix 3 was also studied in HIV-1 (D51A) 14 and HTLV-1 (D54A) 33. In the latter work it was shown that the structure of the D54A mutant is exclusively helical and in contrast to the HTLV-1 wild-type
no β-hairpin was formed. Except of the Asp54, the largest structural changes were observed for the N-terminal residues Pro1-Met17 that form the β-hairpin in the wild-type HTLV CA. Smaller changes were detected for helix 1, the carboxyl terminus of helix 2, helix 5, and helix 6. These results correlate with our data on M-PMV specifically the decrease of the β-sheet content in our CD measurements.

The structural changes induced by the D51A mutation in HIV-1 affect most residues of the β-hairpin and helices 1, 2, 6, and the N-terminal parts of helices 3 and 7. The structural changes connected with the D57A mutation in M-PMV closely resemble those in the D51A mutation in HIV-1 as the positions of helices 5 and 6 in M-PMV NTD are similar to the CA HIV-1 NTD CA helices 6 and 7. In HIV-1, the morphological switch from immature (spherical) to mature (conical) particle is associated with conformational changes accompanying the formation and stabilization of the β-hairpin within helix 3 and 6 of NTD CA. The replacement of D51 in HIV-1 with glutamate, glutamine or asparagine, i.e., more structurally related amino acids compared to alanine, partially restored the ability to assemble both in vitro and intra-cellularly. However, the mutated virus was poorly released and was noninfectious. This indicates indispensable and probably more complex role of this aspartate residue than only the salt bridge formation. The aspartate replacement may also lead to reduced association of RT with the more loosely packed cores as suggested by Tang et al. The β-hairpin stabilization might be common in the retroviruses where morphological switch, from spherical to conical or cylindrical particles occurs. This hypothesis correlates with the finding that in HTLV-1, where such morphological transition does not occur, as both immature and mature particles are spherical, these regions were only minimally perturbed by the salt bridge disruption.

To summarize, we have determined the high resolution structure of the N-terminal domain of the retroviral capsid protein of Mason-Pfizer monkey virus, a member of Betaretro-
virus family, using high resolution NMR spectroscopy. The M-PMV NTD CA solution structure is similar to other retroviral capsid structures solved to date and is characterized by a six-α-helical bundle and an N-terminal β-hairpin. The structure shows that the N-terminal β-hairpin is stabilized by a salt bridge formation between Pro1 and Asp57. Orientation of the β-hairpin, almost parallel to the axis of a six-α-helical bundle, is determined by the electrostatic interactions of its residues to the short helix α5. Besides structure, also dynamics of the N-terminal domain was characterized using the $^{15}$N NMR relaxation data. The structure is relatively rigid, with the exception of a partially flexible N-terminal β-hairpin and loops connecting helices. Compared to the other CA NTD structures, a region reminiscent of the cyclophilin binding loop is shorter, having only a single regular helical motif. In contrast, the corresponding regions of CA NTD structures of most of the other retroviruses contain two short helices. Residues in the range 102-113, where a second helical turn could be formed, show increased flexibility indicating that the measured experimental restraints represent an average value for a broader ensemble of structures present at room temperature. In addition, NMR spectra of mutants ΔP1 NTD CA and D57A CA NTD showed that point mutations of the residues Pro1 and Asp57 dramatically affect the structure within the region of β-hairpin and helices α3 and α5, and lead to destabilization of the β-hairpin. Nevertheless, both mutations when introduced into the M-PMV capsid-nucleocapsid fusion protein did not block the assembly of spherical, immature-like particles. Structural data also explained that the other putative interacting aspartate in position 50 could not participate in formation of the β-hairpin due to its orientation in the helix. This is consistent with the fact that in contrast to the D57A the D50A mutant does not form spherical particles. Both ΔP1 and D57A mutations induced an additional cleavage by the M-PMV protease within helix 2 suggesting different packing of the helical bundle.

**Materials and methods**
**Bacterial constructs**

All DNA manipulations were carried out by using standard subcloning techniques and plasmid DNAs were propagated in *E. coli* DH5α. All newly created constructs were verified by DNA sequencing. The expression vectors for NTDCA\(^{1-140}\) and ΔP1CANC were prepared as described in \(^{22, 36}\). Bacterial expression vectors for D57ACANC were prepared by oligonucleotide-directed *Pfu*-mediated mutagenesis using CANCpET22b template, constructed as described earlier \(^{22}\). The DNA sequences coding ΔP1NTDCA\(^{1-140}\) and D57ANTDCA\(^{1-140}\) were amplified by PCR and inserted into pET22b bacterial expression vector (Novagen), between *Nde*I and *Xho*I sites.

**Protein purification for NMR.**

To define the N-terminal domain of the full length M-PMC capsid protein (1-226) a limited trypsin digestion was performed. A fragment of molecular weight of 16.271Da and N-terminus of PVTET, corresponding to the fragment CA\(^{1-149}\), was identified using MS and N-terminal sequencing. After the initial NMR studies of CA\(^{1-149}\) a non-structured flanking 9 amino acids long C-terminal sequence was removed and the CA protein consisting of 1-140 amino acids was used for NMR measurement. The proteins NTDCA\(^{1-140}\), ΔP1NTDCA\(^{1-140}\) and D57ANTDCA\(^{1-140}\) were expressed and purified as described earlier \(^{36}\). Shortly, the proteins were expressed at high levels in *Escherichia coli* and purified under native conditions by gel chromatography. To achieve \(^{13}\)C and \(^{15}\)N isotopic enrichment the transformed bacterial cells were grown in M9 minimal medium supplemented with \(^{13}\)C D-glucose and \(^{15}\)N ammonium chloride.

**NMR spectroscopy**
The NMR sample consisted of 1.0 mM $^{13}$C,$^{15}$N-labeled M-PMV NTD CA in 50 mM Tris buffer, pH 8.0, containing 150 mM NaCl, 0.25 mM TCEP, and 10% deuterium oxide. The volume of the sample was 550 μl.

All NMR experiments were recorded at 295 K on Bruker Avance 600 MHz equipped with a cryogenic $^1$H/$^{13}$C/$^{15}$N TCI probehead. Resonance assignment of the M-PMV CA NTD was performed using standard triple-resonance and HCCH-TOCSY spectra, as reported previously. The $^{15}$N-edited and $^{13}$C-edited NOESY-HSQC spectra with mixing time of 120 ms were recorded to obtain distance restraints.

The sample described above and a comparable sample aligned using the stretched 4.5% polyacrylamide gel were used for extraction of residual dipolar couplings (RDCs). The IPAP [$^{15}$N, $^1$H]-HSQC, HN[C]-$^3$E and 2D version of the $^{13}$C' detected HCACO experiment were used for the coupling constants measurements.

The $^{15}$N relaxation data were measured on uniformly $^{15}$N-only labeled sample. Longitudinal relaxation time $T_1$, transversal relaxation time $T_2$, and heteronuclear steady-state $^1$H-$^{15}$N NOE were obtained using standard experiments. The $T_1$ and $T_2$ relaxation delays were sampled at 11*, 56, 134, 235*, 381*, 560*, 896, 1344* ms and 15*, 31, 62, 93*, 155*, 217*, 248*, 403 ms, respectively. Values marked with asterisk denote times for which measurement was repeated. The heteronuclear $^1$H-$^{15}$N NOE spectra were acquired with $^1$H saturation composed of train of sine-bell shaped pulses applied at 2,5 kHz field strength for 665 ms. The reference spectrum was obtained using the same conditions, but with the carrier frequency set to 46 kHz off-resonance during saturation period. The relaxation delay was set to 12 s. The rows of the $^1$H-$^{15}$N NOE and reference spectra were recorded in interleaved manner.
The combined chemical shift perturbation (CCSP) was calculated using the following equation:

$$
\Delta \delta_{\text{comb}} = \sqrt{\frac{1}{2} \left( (\delta_{HN(\text{mut})} - \delta_{HN(\text{wt})})^2 + 0.154(\delta_{N(\text{mut})} - \delta_{N(\text{wt})})^2 \right)}
$$

where 0.154 is the weighting factor accounting for different sensitivity of $^1$H and $^{15}$N. All spectra were processed using NMRPipe and analyzed using Sparky (T.D. Goddard and D.G. Kellner, UCSF, San Francisco, CA).

**Distance, dihedral angle, and RDC restraints and structure calculation**

Backbone $\phi$ and $\psi$ torsion angles were derived from the amide H, N, C', C$^\alpha$ and C$^\beta$ chemical shifts using the TALOS software and used as restraints with a minimal error $\pm 20^\circ$. The CANDID 1.1 software package in concert with XPLOR-NIH 2.14 was used for the initial NOE assignment of unambiguously identified NOE cross-peaks. Subsequently, the obtained unambiguous long-range restraints were used as input distances for the ARIA 2.2/CNS 1.2 software to obtain final NOE assignment with floating chirality. During all calculations, the intra-helical and $\beta$-strand hydrogen-bonds were fixed by constraining the O··H distance within the 1.8 Å to 2.3 Å range in the regions identified as regular secondary structure elements based on TALOS prediction and on long-range H$^\alpha$-H$^\alpha$ NOE's. From the measured RDCs, only those exhibiting the internal consistency within the peptide plane and/or in the regular helices were used as structural restraints.

Structure refinement was performed with CNS version 1.2 using the RECOORD water refinement protocol and modified to include the TENSO module of CNS for the RDCs potential term treatment.
NMR relaxation analysis

The backbone amide model-free dynamic parameters were derived using the Lipari-Szabo approach\textsuperscript{58, 59}. The $T_1$, $T_2$ fitting and the model-free analysis was performed using software Relax\textsuperscript{60, 61}. The average steady-state $\text{^{1}H}_{\text{^{15}N}}$ NOE enhancement and associated standard deviation were calculated from three independent measurements. The internuclear distance of the backbone N-H pair was set to 1.02 Å and the nitrogen chemical shielding anisotropy to -160 ppm. The overall tumbling was treated in two different ways, according to the used analysis of the relaxation data (comparison with the hydrodynamic simulation and estimation of parameters of internal motions). For the sake of comparison with the hydrodynamic calculation, the apparent overall correlation time was fitted separately for each residue. For the analysis of internal motions, a rotational diffusion tensor describing tumbling of the whole molecule was applied. The elements of the rotational diffusion tensor were determined using the following procedure. First, based on the analysis of spectral density function values described by Krizova et al.\textsuperscript{25}, a set of residues not influenced by chemical exchange and/or a large extent of internal dynamics was chosen. Second, the initial values of the diffusion tensor were obtained by the program Tensor2\textsuperscript{62}. Several runs of the program were performed and residues exhibiting the largest differences between the back calculated and experimental ratio $R_2/R_1$ in the previous run were excluded from the next iteration until the fitted values of the rotational diffusion tensor elements were accepted by the $\chi^2$ test. Third, program Relax\textsuperscript{60, 61} was run to optimize parameters of the internal motions (namely $S^2$ and $\tau_\varepsilon$) having the rotational diffusion tensor initially fixed. In the subsequent calculations, the obtained parameters of the internal motions were kept constant and the rotational diffusion tensor was optimized. Those two successive Relax runs were repeated until a convergence was reached. The refined rotational diffusion tensor was then employed in a model-free analysis of all residues for which reliable relaxation data were obtained. One of the five
standard models of the internal motion was selected using the Akaike information criteria\(^{60, 61}\).

Hydrodynamic calculations

The HYDRONMR\(^{26, 63}\) was used for calculation of hydrodynamic parameters. Temperature of 295K, viscosity of 9.55·10^{-4} kg·m^{-1}·s^{-1}, and atomic element radius of 3.2 Å were used in the simulations. In order to minimize the error introduced by the beads position setting algorithm, the lowest value minimum radius of beads in the shell, which does not exceed the limiting number of 2000 beads in the shell, was determined (1.04 Å) and set in the calculations. The uncertainty of the obtained data was assessed from 36 repeated simulations for varied starting orientations of the molecule.

Circular Dichroism

Circular dichroism spectra were recorded at room temperature using a Jasco J-810 spectrometer (Jasco, Tokyo, Japan). Data were collected from 185 to 260 nm, at 100 nm/min, 1 s response time and 2 nm bandwidth using a 0.1 cm quartz cuvette containing studied protein in 50 mM potassium phosphate buffer and 150mM KClO\(_4\) (pH=8.0). Each spectrum shown is the average of ten individual scans and was corrected for absorbance caused by the buffer. CD data were expressed in terms of the mean residue ellipticity (\(\Theta_{MRE}\)) using the equation:

\[
\Theta_{MRE} = \frac{\Theta_{obs} \cdot M_w \cdot 100}{n \cdot c \cdot J}
\]

where \(\Theta_{obs}\) is the observed ellipticity in degrees, \(M_w\) is the protein molecular weight (15455.10 g/mol), \(n\) is number of residues (140), \(l\) is the cell path length (0.1 cm), \(c\) is the protein
concentration (0.135 mg/ml and 0.138 mg/ml for D57A CA NTD; 0.138 mg/ml for WT CA NTD; and 0.191 mg/ml and 0.109 mg/ml for ΔP1 CA NTD) and the factor 100 originates from the conversion of the molecular weight to mg/dmol.

Secondary structure content was calculated from the spectra using Self Consistent method Selcon3 \(^{64-66}\) implemented in the program DICROPROT (http://dicroprot-pbil.ibcp.fr) \(^{67}\).

**Bacterial expression and purification of the wt and mutant CANC**

Bacterial expression and purification of recombinant proteins were carried out as described previously \(^{22}\) with some modifications. Bacterial pellet obtained from 400 ml of Luria-Bertani medium harvested 4 hours post induction with 0.4mM IPTG was resuspended in 12 ml of lysis buffer A (50mM Tris-HCl, 150mM NaCl, 1mM EDTA, pH 8) containing 0.1% 2-mercaptoethanol, Pefablock (Roche) and cocktail of inhibitors (Sigma). Cell lysate was stirred for 30 min in 4°C, sonicated on ice and treated with sodium deoxycholate (final concentration of 0.1 %) in 4°C for 30 min. After centrifugation at 10,000xg for 15 min the proteins were solubilized from the pellet by the addition of 10 ml of buffer A containing 0.5% Triton X-100, 1M NaCl and centrifuged 15 min at 10,000xg. The pellet was solubilized again in 10 ml of buffer A containing 0.1% Triton X-100, 0.1% 2-mercaptoethanol, 1 M NaCl and centrifuged 15 min at 10.000xg. Proteins released into the supernatant were dialyzed against buffer Z (50mM phosphate, 500mM NaCl, 0.05% 2-mercaptoethanol, pH 7.5) overnight at 4°C. Dialed material was loaded on the top of a Zn- chelating fast flow Sepharose chromatography column equilibrated in buffer Z. The bound proteins were eluted with 2M NH₄Cl. The fractions containing desired proteins were dialyzed against the buffer H (10mM phosphate, 0.15mM NaCl, pH 7.3) and loaded on the Heparin-Sepharose CL-6B column (15 ml). The bound proteins were eluted by a salt gradient (100mM to 2M), the fractions containing the pure protein were pooled, concentrated to 1 to 2mg/ml and stored at -70°C.
In vitro assembly

60 μg aliquot of purified ΔP1CANC and CANCD57A proteins was mixed with 6 μg of oligodeoxyribonucleotides (76-mer) and dialyzed against the assembly buffer (50mM Tris-HCl, 100mM NaCl, 1μM ZnCl2, pH 8) overnight at 4°C using 1kDa dialysis tube (Spectrapor). Particles formed during assembly were negatively stained with 4% sodium silicotungstate (pH 7.2) on carbon-coated grids and studied by transmission electron microscopy JOEL JEM 120.

Proteolytic cleavage

Cleavage of CANC proteins with M-PMV protease was performed as published previously.\textsuperscript{29}

Accession numbers

Coordinates and NMR restraints were deposited in the Protein Data Bank with accession number 2kgf. The $^{15}$N NMR relaxation data were deposited in Biological Magnetic Resonance Bank with accession number 16234.

Acknowledgments

We thank Romana Cubínková for excellent technical assistance. The work was supported by grants of the Ministry of Education, Youth and Sports of the Czech Republic MSM0021622413 to J. Ch, R.Ch., and L.Ž, LC06030 to P.K. and V.S., LC545 to P.M., LC7017 to J.Ch, M6138896301, the grant of Grant Agency of the Czech Republic 204/09/1388 to MR, the research projects from the Ministry of Education of the Czech Republic: 1M0508 and Z 40550506 and by a grant SCO/06/E001 (EUROCORES) from the
Czech Science Foundation, grants of Czech Ministry of Education 1M6837805002, MSM 6046137305 and grant CA 27834 from the National Institutes of Health and Z 4055905 I.P.
Figure Legends.

Figure 1.
Stereo diagram showing backbone wire representations of 50 structures of M-PMV CA NTD with the lowest energy. The structures are superimposed over the backbone atoms (Cα, N, C') of secondary structure elements. The green to red gradient color coding of the ensemble represents the RMSD of the experimental harmonic mean rotation correlation time of individual structures vs. the values predicted by HydroNMR (green, RMSD=4.3 ns; red, RMSD=12.9 ns).

Figure 2.
Stereo view of the ribbon representation of the M-PMV CA NTD lowest energy structure. Secondary structures are depicted in cyan (β-hairpin), orange (helix α1), magenta (helix α2), blue (helix α3), green (helix α4), yellow (helix α5), and red (helix α6).

Figure 3.
Dynamics of P-PMV CANT studied by $^{15}$N NMR relaxation. A) relaxation times T$_1$; B) relaxation times T$_2$; C) steady-state $^{1}$H–$^{15}$N NOE; D) generalized order parameter S$^2$ (crosses, residues without signs of conformational exchange; open circles, residues with order parameter affected by conformational exchange); E) harmonic mean rotation correlation time $\tau_m$ calculated from experimental T$_1$, T$_2$, and $^{1}$H–$^{15}$N NOE values (crosses) and predicted by HydroNMR for 36 repeated simulations with varied starting orientations of the molecule (cyan diamonds). Secondary structure elements are marked by color bars in the bottom panel with the same color coding as in Figure 2.
Figure 4.

Projection of combined chemical shift perturbation (CCSP) of (A) ΔP1 and (B) D57A onto NMR structure of M-PMV CA NTD. Residues are color coded in blue-green-red. Blue, residues with CCSP=0; red, residues with CCSP≥0.1ppm. Unassigned and proline residues of wt CA NTD are marked in gray, while residues assigned for the wild-type but not for the mutant are marked by magenta.

Figure 5.

Comparison of ΔP1CANC and CANCD57A virus-like particles using electron microscopy.

Purified protein samples of A) ΔP1CANC and B) CANCD57A were mixed with nucleic acid and dialyzed against the assembly buffer, negatively stained and examined by electron microscopy.

Figure 6.

Analysis of proteolytic cleavage of CANC, ΔP1CANC and CANCD57A.

Purified proteins (panel A) were incubated overnight with M-PMV protease (panel B) and the cleavage products were analyzed by SDS-PAGE and Western blotting, using rabbit anti-M-PMV CA antibody.

Figure 7.

Comparison of M-PMV CA NTD with retroviral structure homologues. (a) NMR structure of M-PMV (2kgf), (b) X-ray structure B-MLV (3bp9), (c) NMR structure from HIV-1 (1gwp), (d) X-ray structure from HIV-1 (1ak4), (e) NMR structure from HTLV-1
(1g03), (f) X-ray structure from RSV (1en9), (g) X-ray structure from JSRV (2v4x), (h) NMR structure from RSV (1d1d), and (i) X-ray structure from EIAV (1eia).

Figure 8.
The detail of the electrostatic interactions between the β-hairpin (residues 1,14,16) and the helices α3 (residue 57) and α5 (residues 111, 117). The interacting residues are shown as stick models, while the secondary structure elements are colored as in Figure 2. The surfaces of positively and negatively charged residues are colored in blue and red, respectively, while hydrogen bonds are shown as dashed black lines.
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Table 1: Statistics of NMR structure determination for 10 lowest energy structures

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<th>Structural statistic</th>
<th>Value</th>
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<td>Hydrogen bonding restraints</td>
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Table 2: Prediction of protein secondary structure based in measured CD spectra

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