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Oligomerization and assembly of the matrix protein of Borna disease virus

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Abstract The matrix protein M of Borna disease virus (BDV) is a constituent of the viral envelope covering the inner leaflet of the lipid bilayer. BDV-M was expressed as recombinant protein in *Escherichia coli*, purified to homogeneity and structurally analyzed. Recombinant M (i) forms non-covalently bound multimers with a Stoke's radius of 35 Å estimated by size exclusion chromatography, (ii) consists of tetramers detected by analytical ultracentrifugation, and (iii) appears by electron microscopy studies as tetramers with the tendency to assemble into high molecular mass lattice-like complexes. The structural features suggest that BDV-M possesses a dominant driving force for virus particle formation.

Keywords: Borna disease virus; Matrix protein quaternary structure

1. Introduction

Viral matrix proteins are constituents of most enveloped viruses forming a layer at the inner side of the viral envelope. Matrix proteins attract increasing interest because they are the most abundant structural components of the virus envelopes and play a critical role in the process of virus assembly and budding at the plasma membrane. They are also assumed to be responsible for viral shape maintenance. It is generally accepted that matrix proteins play an important role in interaction with ribonucleoprotein (RNP) and with intraviral amino acid sequences of envelope glycoproteins [1]. Furthermore, matrix proteins are involved in regulation of virus replication and in RNP transport to the cellular membrane for generation of virus progeny as, for instance, demonstrated with the influenza virus matrix protein [2]. Currently, increasing data are available which indicate that matrix proteins are responsible for an active release of virus particles. This was shown, for example, for HIV, Ebola- and Lassavirus among other enveloped viruses [3–5]. Structures of some viral matrix proteins were elucidated at the atomic level, e.g. HIV, influenza virus, and two representatives of the order *Mononegavirales*, Vesicular Stomatitis virus (VSV) and Ebola virus [6–9]. When the Ebola virus matrix protein is assembled at the lipid membrane it changes its conformation and acquires specific RNA binding characteristics [10–12]. In order to elucidate whether viral matrix proteins are arranged principally in similar or different types of arrays it is of interest to analyze several various enveloped viruses. In this respect, we investigated the matrix protein M of Borna disease virus (BDV).

BDV is the only representative of the *Bornaviridae* family within the order *Mononegavirales*, which also includes measles-, rabies-, Ebola-, Marburg viruses and numerous other important pathogenic viruses. BDV is the etiological agent of Borna disease (BD), a sporadically occurring, progressive viral encephalomyelitis that primarily affects horses and sheep. During the last decades, infection of BDV was observed in an increasing number of domestic and zoo animals worldwide. Serological evidence of BDV infection has been found in numerous countries throughout the world. After an incubation period lasting a few weeks to several months, BDV infection can cause locomotor and sensory dysfunctions followed by paralysis and death. BD is the result of virus-induced immunopathological reactions. BDV-specific antibodies and viral RNA have been found in humans with various psychiatric diseases. But it is still unclear, whether BDV causes any illness in man [13,14].

BDV is an enveloped, single-stranded RNA virus containing the smallest genome of negative sense among the *Mononegavirales* which also reflects the smallest virus-coded proteins, especially the matrix protein M. BDV is characterized by nuclear transcription and replication and by using RNA splicing machinery of host cells which is unique within this virus order. The BDV genome consists of at least six open reading frames (ORFs). They code for p40/38 nucleoprotein (N) (ORF I), p24 phosphoprotein (P) (ORF II), p16 matrix protein (M) (ORF III), p57 glycoprotein (GP) (ORF IV), RNA polymerase (L) (ORF V), and p10 protein (X) (ORF VI). GP, previously designed as gp84, gp94 or G, is the N-glycosylated precursor of p57. GP is proteolytically cleaved into the complex GP-N/GP-C which is present in the mature virus particles as spikes [15]. Proteins N, P, X and L are associated with the RNA genome forming the RNP-complex [16]. Previously, it was assumed that p16 of ORF III encodes a viral glycoprotein (gp16) [17,18]. However, a reinvestigation of the gene product of ORF III revealed that p16 is a non-glycosylated membrane protein which can be detached from lipid bilayers without detergents. These characteristics showed that p16 is the viral matrix protein M [19]. BDV possesses the smallest matrix protein with a molecular mass of 16 258 kDa among all

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Abbreviations: BD, Borna disease; BDV, Borna disease virus; M, matrix protein; SDS–PAGE, Sodium dodecylsulfate polyacrylamide gel electrophoresis

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negative-stranded, enveloped RNA viruses. It has no apparent sequence similarity to other matrix proteins. Recombinant BDV-M was expressed in *Escherichia coli* (*E. coli*), purified and crystallized [20]. The elucidation of the X-ray structure of BDV-M is currently in progress.

Here, we report how M oligomerizes and is assembled to a high-order protein layer by different physical–chemical protein analyses.

2. Materials and methods

2.1. Cloning, expression, and purification of the BDV-M protein

The full-length BDV-M gene was isolated from viral RNA of BDV-strain He/80 and cloned into the expression vector pMAL-c2 (New England Biolabs) by using a RT-PCR technique as described before [20]. The desired maltose binding protein fused to BDV-M (MBP-BDV-M) was obtained after transformation of *E. coli BL21* cells with the recombinant vector pMAL-c2/BDV-M. MBP-BDV-M was purified from lysed *E. coli* cells by affinity chromatography using amylose bound to carrier-beads. The BDV-M moiety containing N-terminally the hexapeptide ISEFGS due to the cloning strategy was cleaved off from MBP-BDV-M by factor Xa (Amersham Biosciences). BDV-M was further purified to homogeneity by using MonoS HR 5/5 chromatography and size exclusion chromatography via a HiLoad 16/60 Superdex 75 column and Superdex 200 HR 10/30 column, respectively (Amersham Biosciences). All chromatography applications were performed with an ÄKTA™ FPLC System (Amersham Biosciences). Finally, purified BDV-M was concentrated to 5 mg per ml by using Centricon-10 (Millipore).

2.2. Electrophoresis of BDV-M under native and denaturating conditions

Separation of the basic BDV-M protein under native conditions was performed on a 15% polyacrylamide gel in the presence of 1.6% acetic acid adjusted with KOH to pH 4.0. The separation gel was overlaid by a 3.75% stacking polyacrylamide gel containing 0.54% acetic acid adjusted to pH 4.0. The M protein sample was diluted in sample buffer consisting of 40 mM β-alanine adjusted with acetic acid to pH 4.0, containing 10% glycerol and 0.04% methylgreen. Gel electrophoresis was performed at 150 V for 3 h at room temperature [21]. Electrophoretic separation of BDV-M under denaturing conditions was performed on a 15% polyacrylamide gel containing 0.1% SDS and ß-mercaptoethanol after heating of the protein M sample at 96 °C for 5 min [22].

2.3. Determination of stoke radius of protein M by size exclusion chromatography

Protein M fractions of unknown molecular mass were dissolved in separation buffer containing 50 mM HEPES, 150 mM NaCl, pH 7.6 and passed through a Superdex 200 HR 10/30 column according to manufacturer's instructions using an ÄKTA FPLC System (Amersham Biosciences). Protein standards from the Low and High Molecular Weight Gel Filtration Calibration Kit (Amersham Biosciences) were separated under identical conditions. Each eluted single protein peak was monitored by measurement of optical density at 280 nm. The collected data were presented as the plot of $(-\log K_{av})^{1/2}$ versus Stoke's radius according to the Laurent and Killander solution [23].

2.4. Chemical cross-linking of oligomerized protein M

Purified BDV-M (5 μg/μl) was supplemented with 0.2 M triethanolamine pH 8.0. Dimethyl pimelinimiate-HCl (DMP, Sigma) was freshly dissolved in 0.2 M triethanolamine to yield a 0.5 mM DMP solution which was adjusted to pH 8.0 by addition of 0.1 N NaOH. 5 μg (1 μl) BDV-M (pH 8.0) was mixed with 2 μl of DMP solution (pH 8.0) and incubated for 10 min at 10 °C. Thereafter the cross-linking reaction was quenched by addition of 1 μl of 2.2 M triethanolamine, mixed with 4 μl Laemmli 4× sample buffer prior to subjection to 15% SDS–PAGE. The protein was analyzed by Coomassie Brilliant Blue R250 staining.

2.5. Matrix-assisted laser desorption ionisation-time of flight mass spectrometry

Purified BDV-M protein samples were desalted by NAP-10 columns (Amersham-Biosciences), and about 1 μMol of each protein sample was embedded in saturated sinapic acid in 0.1% (v/v) trifluoroacetic acid, 67% (v/v) acetonitril. For each spectrum, at least 100 single shots were totalized. The spectra were determined with a Voyager DE RP from PE-Biosystems.

2.6. Analytical ultracentrifugation

Sedimentation analyses were conducted using an analytical ultracentrifuge Optima X-LA (Beckman Instruments, Palo Alto, CA) equipped with two channel cells and an AnTi 50 rotor. The protein concentration varied between 10 and 500 μg per ml. Dilutions of recombinant M were performed with 50 mM HEPES buffer pH 7.6 containing 150 mM NaCl. Centrifugation velocity was performed at 40 000 rpm at 20 °C. Centrifugation equilibrium was measured at 12 000 rpm, 20 °C. The samples were monitored at 230 and 280 nm, respectively. The apparent *s*-values and molecular masses were calculated using the software provided by Beckman Instruments. The dependence of the apparent *s*-values on the protein concentration was fitted according to Luther and co-workers [24] assuming a tetramer octamer equilibrium.

2.7. Electron microscopy

Indirect carbon support films were prepared as described previously [25]. Purified full-length recombinant M protein was applied to the carbon prior to negative staining with an aqueous solution of 4% (w/v) uranyl acetate. The stained specimen was mounted onto a 400 mesh copper grid. Each specimen was observed in a Zeiss EM T109 electron

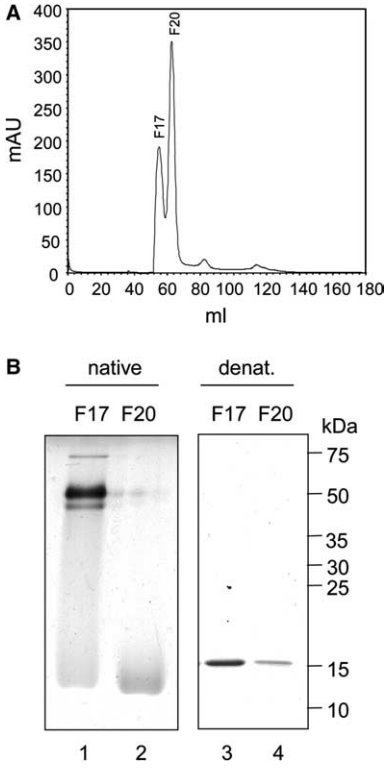

Fig. 1. (A) Gel filtration chromatography of recombinant BDV-M. Elution profile of purified M passed through Superdex 75 column, UV adsorption at 280 nm. (B) Native and denaturating polyacrylamide gel electrophoresis. Fraction F17 and F20 from Superdex 75 column subjected to native (lanes 1 and 2) and 15% SDS–PAGE (lanes 3 and 4). Positions of molecular weight marker proteins are indicated for electrophoretic separation in polyacrylamid gels under denaturating conditions.

microscope operated at an acceleration voltage of 80 kV. Electron micrographs were recorded at a calibrated magnification (48 200×, by using a cross-ruled diffraction grating replica with 2160 lines/mm).

3. Results

3.1. Purification of BDV-M protein

Isolation of BDV-M in pure form is not feasible from virus preparations because BDV propagation in cell cultures is very inefficient. Furthermore, the BDV particles are very instable, thus not allowing extensive purification of virus preparations. Therefore, studies of oligomerization and assembly of full-length BDV-M under physiological conditions were performed with *E. coli* recombinant BDV-M. The M gene was obtained from permanently BDV infected mammalian cell cultures by RT-PCR technique. Recombinant BDV-M was then expressed in *E. coli* as a fusion protein linked to maltose binding protein (MBP). After BDV-M was cleaved off from the fusion protein, it was subjected to several chromatographic purification steps including maltose resin, ion exchange chromatography, and size exclusion chromatography using a Superdex 75 gel permeation column. BDV-M was observed in two peak maxima, i.e. in fractions 17 and 20 (Fig. 1A). The 280–260 nm ratio of about 1.1 indicated that these fractions might contain oligonucleotides incorporated in M oligomers, since VP 40 of Ebola virus was found to bind a triribonucleotide [12]. Fractions 17 and 20 of the Superdex 75 column were subjected to gel electrophoresis under native and denatured conditions. Fraction 17 shows predominantly a form with poor electrophoretic mobility, whereas fraction 20 exhibits a higher mobility indicating a smaller complex (Fig. 1B, lane 1 and 2). In both fractions only one protein band with a molecular mass of 16 kDa under denaturing conditions was obtained (Fig. 1B; lane 3 and 4). This result demonstrated that BDV-M was purified nearly to homogeneity, but it exist in at least two multimeric forms.

3.2. Homooligomerization of BDV-M

Purified BDV-M of fraction 20 of the Superdex 75 column was also analyzed by mass spectrometry in order to clarify the molecular mass of M in this fraction. The matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) analysis of M of fraction 20 revealed the maximal molecular masses of 16 875 for M (Fig. 2A), which differs only by 3.5 Da from the calculated molecular mass of 16 878.5.

Both fractions of the Sephadex 75 column were subjected to a re-chromatography on a Superdex 200 HR 10/30 column, calibrated with molecular mass markers. Re-chromatography resulted in a peak with the same elution volume for both fractions (Fig. 2B). The Stoke's radius of protein M was calculated

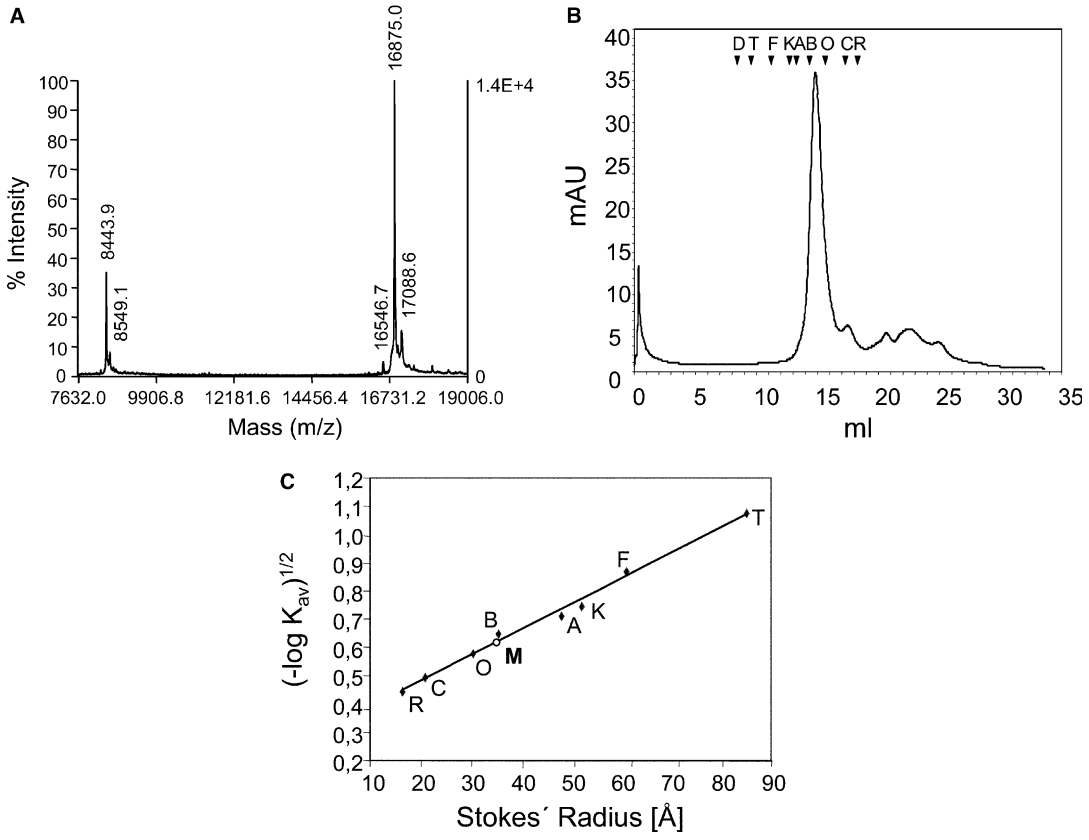

Fig. 2. (A) Mass spectrum of BDV-M. MALDI-TOF-MS spectrum is shown for Superdex 75 fraction F20. Spectrum was collected with sinapic acid as matrix. The 100% scale of the relative intensities refers to 14 000 counts. (B) Rechromatography of F20 on Superdex 200 column and standard proteins. UV adsorption at 280 nm, arrows indicate elution volumes of the following standard proteins: R: ribonuclease A (13.7 kDa, 16.4 Å), C: chymotrypsinogen A (25 kDa, 20.9 Å), O: ovalbumin (43 kDa, 30.5 Å), B: albumin (67 kDa, 35.5 Å), A: aldolase (158 kDa, 48.1 Å), K: catalase (232 kDa, 52.2 Å), F: ferritin (440 kDa, 61 Å), T: thyroglobulin (669 kDa, 85 Å) and D: Blue Dextran 2000. (C) Determination of the Stoke's radius of M from fraction 20 (see B). The Stoke's radius of M was calculated as 35 Å.

by the elution volumes of fraction 20 and the elution volumes of the marker proteins indicated in Fig. 2B. The Stoke's radius of M was determined as 35 Å (Fig. 2C), that would fit a BDV-M tetramer.

M protein from fraction 20 was already used for crystallization of M [20]. Since no higher molecular mass species were detected for M by mass spectrometry, oligomer formation of M in fraction 17 and 20 of Superdex 75 column must be based on non-covalent interactions. Taken the data of gel permeation chromatography, gel electrophoresis and mass spectrum analyses together, it is reasonable to speculate that protein M exist in a non-covalently oligomeric form.

3.3. Sizing of oligomerized BDV-M

Analytical ultracentrifugation analysis is the appropriate method to accurately determine the molecular mass of the oligomerized BDV-M independently of gel permeation chromatography. The two distinct peaks from the gel filtration of BDV-M on Sephadex 75 column were analyzed by analytical ultracentrifugation. The protein of the elution peak fraction 20 consists of a homogeneous species of Mr = 65 200 ± 1850 (Fig. 3A). This molecular mass fits well to a tetrameric state of BDV-M with a theoretical mass of 67 500. Neither the apparent molecular mass nor the *s*-value of sedimentation velocity (s_{app} = 3.57 S) were dependent on the protein concentration at a range of 0.05–0.5 mg/ml, proving the tetramer as the stable structural unit of this protein.

In contrast to this result, the viral protein of the gel filtration peak 17 showed a faster apparent sedimentation velocity and a significant variation of the *s*-value with protein concentration (Fig. 3B). This dependency could be fitted to a tetramer–octamer equilibrium and a dissociation constant of K_D = 0.6 μM. However, the data do not rule out that at higher protein concentrations (>0.5 mg/ml) even larger association states of the tetrameric unit might occur, which are built by the stable tetrameric unit of BDV-M.

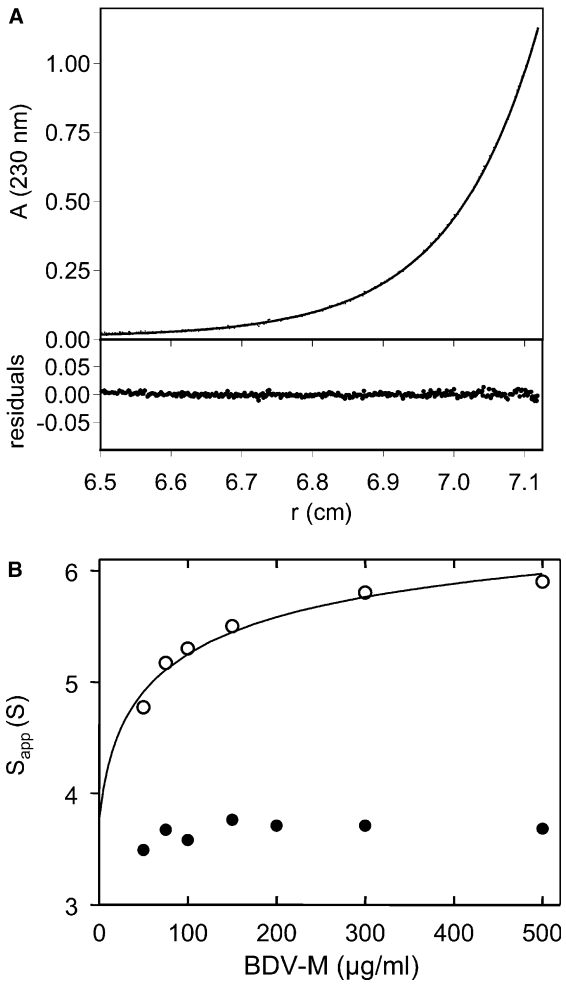

Fig. 3. Analytical ultracentrifugation. (A) Molecular mass of BDV-M. Molecular mass determination of BDV-M F20 was performed by sedimentation equilibrium measurement at 10 000 rpm, 20 °C in an analytical ultracentrifuge. The protein concentration was 0.3 mg/ml. The experimental data (dots) could be fitted (solid line) to a homogeneous species with a calculated mass of 65 160 Da, representing a stable tetramer. (B) Association state of BDV-M. The dependence of the apparent *s*-value of sedimentation of BDV-M on the protein concentration was measured at 40 000 rpm, 20 °C. Whereas the sedimentation velocity of BDV-M F20 was independent of the protein concentration (•), the species BDV-M F17 displayed an increase in the *s*-value with increasing concentration (○). These data could be described by a tetramer–octamer equilibrium with a dissociation constant of 0.6 μM (solid line), assuming a starting value at very low protein concentrations identical to that of BDV-M F20.

3.4. Assembly of oligomeric forms of BDV-M

In order to answer the question whether higher oligomeric forms of BDV-M exist, M fractions were analyzed by chemical cross-linking and electron microscopy. Cross-linking of purified BDV-M fraction 20 of the gel permeation chromatography shown in Fig. 1 resulted in a slightly different SDS-denatured state of the monomer, leading to a shift in electrophoretic multimers indicating preformed M of higher order (Fig. 4A). Therefore, it was interesting to analyze whether unspecified oligomers accumulate or regular arrays of BDV-M may be visible. Although the molecular size of M is at the lower limit for electron microscopic analyses of M-oligomers, these studies were performed with purified recombinant BDV-M of fraction 20 and presented in two overviews (Fig. 4B-1 and B-6). The majority of the particles was homogeneous and formed elementary units of tetramers which exhibit a characteristic central hole. The dimension of a tetramer is consistent with the molecular mass of 16 kDa for monomers and about 60 kDa for tetramers [26]. However, at this stage of optical resolution it cannot be excluded that M-tetramers may not be distinguishable from octamers formed by head-to-head dimerized tetramers (Fig. 4B-3 to B-5). Furthermore, the tetramers of M are partially arranged to short regular two-dimensional various geometric features in several areas of the overview micrograph (Fig. 4B1 and B-6). Several possibilities of those tetrameric BDV-M arrangements are schematically depicted (Fig. 4B-7). In general, the analysis of proteins by electron microscopy was developed for macromolecules of the size of haemoglobin, for instance, but the results obtained with BDV-M were convincing. However, based on the limited resolution the particle analysis does not unambiguously show which types of lattices prevail.

In conclusion, our data clearly indicate that *E. coli* recombinant M protein of BDV exists as stable non-covalently linked tetramers which rudimentarily assemble to net-like structures.

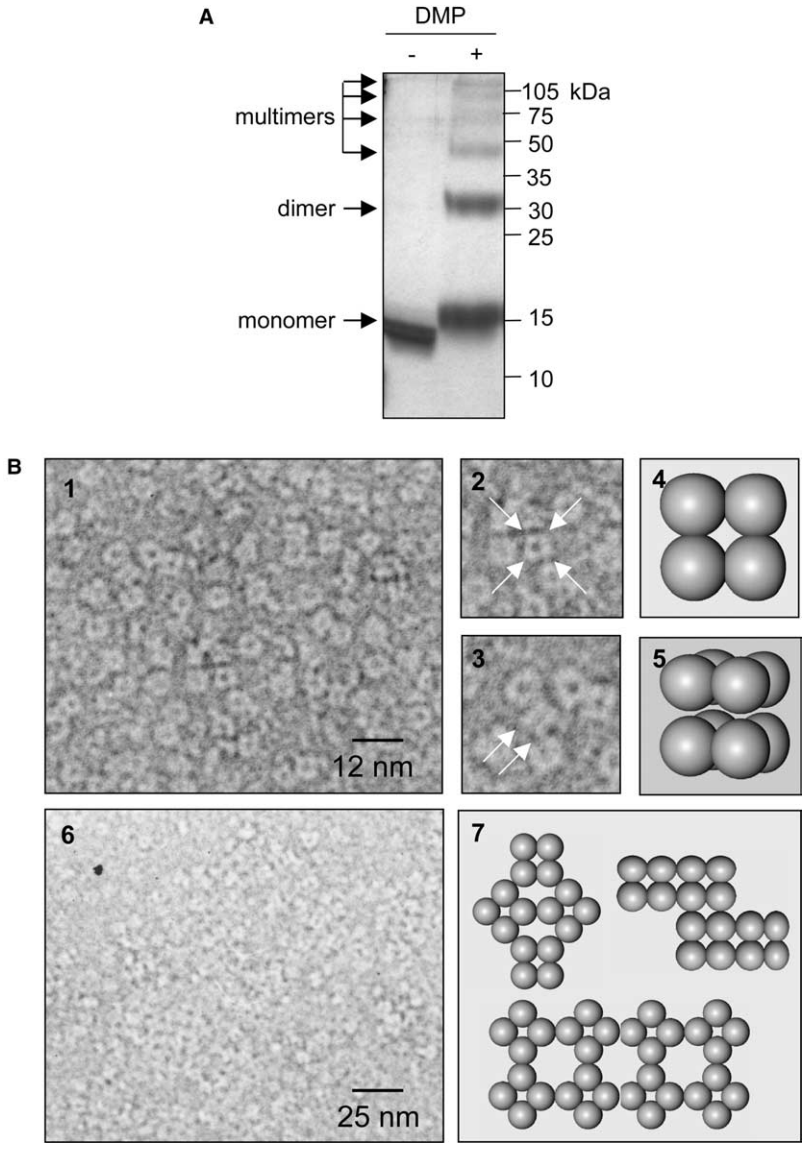

Fig. 4. (A) Chemical cross-linking of recombinant BDV-M. Electrophoresis of recombinant BDV-M of fraction 20 of Sephadex 75 column chromatography is shown before and after chemical cross-linking. Purified M was kept untreated (−) or treated (+) with the cross-linker DMP and subjected to 15% SDS–PAGE. The proteins were stained with Coomassie Brilliant Blue. The positions of recombinant standard proteins are indicated. (B) Electron microscopic analysis of purified, full-length BDV-M. M was negatively stained with 4% (w/v) uranyl acetate. Overview of M oligomers (1, 6), typical tetrameric M (2), possible octamer (3), models of tetramer (4) and octamer (5), M forming characteristic rudimentary lattices (6), and models depicted from (6) of regular two-dimensionally assembled M (7).

4. Discussion

Virus assembly requires the transport of many viral components to the assembly site where they are concentrated and segregated from cellular components so that interactions between viral components become more and more likely. Primary weak interactions between single or preformed viral components enhance and amplify the co-operations among them. Assembly of enveloped viruses depends on selective association of viral proteins at two-dimensional lipid bilayers [27]. In this assembly process viral matrix proteins play a central role. They exhibit a scaffolding layer lined on the inner lipid surface of the virus envelope. In recent years, the structure and function of viral matrix proteins in connection with the late step of virus budding from cellular membranes became of high interest [28]. BDV has the smallest genome among the *Mononegavirales* and accordingly the shortest amino acid sequences of the corresponding gene products. It is therefore a suitable model for understanding the function of the BDV-M and other interacting BDV proteins at the atomic level. The BDV matrix- and nuclear protein were crystallized and the crystal structure of the nucleoprotein was successfully elucidated, the first one among all negative single-stranded RNA viruses [20,29].

Previously, we demonstrated that recombinant BDV-M isolated from the soluble cytoplasmic fraction of *E. coli* has the same electrophoretic mobility as M of BDV-infected material under denaturing conditions (data not shown). Here, we show that BDV-M forms tetramers as the most stable elementary unit of M under physiological conditions. This is independently

shown by gel filtration, electron microscopy and analytical ultracentrifugation. The lack of cysteine residues in BDV-M excludes oligomers which might be formed by disulfide bonds. Denaturing gel electrophoresis of M reveals a band with about 15 kDa, indicating a monomer of BDV-M. Chemical cross-linking experiments of M under physiological conditions shift the monomer to dimers, tetramers, and other multimers of M reflecting its tendency for self-assembly. In addition, MALDI-TOF analysis reveals non-covalently bound monomers of all forms of recombinant BDV-M. Since the mass spectroscopy of the different BDV-M size-species obtained by gel permeation chromatography resulted always in the identical mass of M, only non-covalent bonds are responsible for the diverse association behavior of M.

Interestingly, different size-species of M only appeared after long incubation at high salt concentration in two fractions of the Mono S column. The re-chromatography of each of the two fractions through a Mono S column results in two peaks again. These observations indicate that equilibrium between tetramers and octamers of BDV-M exists due to its weak reversible binding forces, which is also supported by the ultracentrifugation studies.

Our electron microscopic studies independently demonstrate the tendency of BDV-M to form stable tetramers and extended complexes based on lateral contacts under physiological conditions. Our previous findings of the cubic space group I432 of the M-crystals is also consistent with this view of a higher ordered BDV-M assembly based on tetrameric elementary units [20]. The ability to form stable non-covalent tetramers that are capable of either forming two- and three-dimensional lattices implicate that BDV-M may be the driving force for virus particle formation.

Whereas M proteins of VSV and influenza virus are monomeric in solution or tend to form very large aggregates [30,31], BDV-M forms multimers in solution like Ebola matrix protein. The crystal structure of Ebola virus VP40 exhibits disc-shaped octamers which are formed by four antiparallel homodimers of the N-terminal domain [12]. The combination of x-ray crystallography on viral matrix proteins and electron microscopic images of negatively stained particles has proved to be a powerful approach to clarify the architecture of the matrix protein layer beneath the lipid layer of a few envelope viruses. At least two different principles have been discerned for matrix protein assisted shape control: (i) the multimerization of a regular array of oligomers to form a net, as suggested for VP40 of Ebola virus, and (ii) a repetitive addition of staggered oligomers to form a flexible ribbon forming a helix as found for influenza virus M1. The data presented here suggest that BDV-M may most likely follow the same rules as found for Ebola virus VP40 matrix formation. It is yet not clear, whether BDV-M binds oligonucleotides as it was found with the Ebola virus matrix protein VP40 which binds specific trinucleotides [32].

Interestingly, the nucleoprotein N [29] and the M of BDV form tetramers in solution. Physiological function of tetramerization of both proteins and their correlation will be further investigated.

In conclusion, we could demonstrate that recombinant BDV-M forms stable tetramers which have the tendency to assemble to geometric regular two-dimensional structures, indicating that M of BDV is the driving force for virus particle formation.

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