JOURNAL OF VIROLOGY, Jan. 1995, p. 613–617
0022-538X/95/$04.00+0

Vol. 69, No. 1

Disulfide Bonds between Two Envelope Proteins of Lactate Dehydrogenase-Elevating Virus Are Essential for Viral Infectivity

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Received 11 July 1994/Accepted 13 October 1994

Disulfide bonds were found to link the nonglycosylated envelope protein VP-2/M (19 kDa), encoded by open reading frame 6, and the major envelope glycoprotein VP-3 (25 to 42 kDa), encoded by open reading frame 5, of lactate dehydrogenase-elevating virus (LDV). The two proteins comigrated in a complex of 45 to 55 kDa when the virion proteins were electrophoresed under nonreducing conditions but dissociated under reducing conditions. Furthermore, VP-2/M was quantitatively precipitated along with VP-3 in this complex by three neutralizing monoclonal antibodies to VP-3. The infectivity of LDV was rapidly and irreversibly lost during incubation with 5 to 10 mM dithiothreitol (>99% in 6 h at room temperature), which is known to reduce disulfide bonds. LDV inactivation correlated with dissociation of VP-2/M and VP-3. The results suggest that disulfide bonds between VP-2/M and VP-3 are important for LDV infectivity. Hydrophobic moment analyses of the predicted proteins suggest that VP-2/M and VP-3 both possess three adjacent transmembrane segments and only very short ectodomains (10 and 32 amino acids, respectively) with one and two cysteines, respectively. Inactivation of LDV by dithiothreitol and dissociation of the two envelope proteins were not associated with alterations in LDV's density or sedimentation coefficient.

In genome size (14.1 kb) and morphology, lactate dehydrogenase-elevating virus (LDV) resembles flaviviruses and togaviruses (20) and, on this basis, had been tentatively placed in the family *Togaviridae* (25). Like these viruses, LDV virions have a diameter of 55 to 65 nm and a cubical nucleocapsid core of 25 to 35 nm in diameter and possess a single-stranded RNA genome of positive polarity (4, 25). However, the LDV genome organization and replication are different from those of flaviviruses and togaviruses and resemble those of coronaviruses (6, 14, 17, 25). Similar to replication of coronaviruses, replication of LDV involves the formation of a 3′-coterminal nested set of seven subgenomic mRNAs (6, 17, 25). It has been suggested that LDV along with the related viruses equine arteritis virus (EAV), simian hemorrhagic fever virus, and porcine reproductive and respiratory syndrome virus be placed in an as yet unnamed new family (25).

The structural proteins of LDV and other members of the group also differ in size, nature, and organization from those of togaviruses (4, 20, 25). Four structural proteins have been identified for viruses in this new family. They are (i) a highly basic nucleocapsid protein (12 to 14 kDa; VP-1 or N; encoded by open reading frame [ORF] 7), (ii) a nonglycosylated 18- to 19-kDa envelope protein (VP-2 or M; encoded by ORF 6), (iii) a primary envelope glycoprotein encoded by ORF 5, and (iv) a minor envelope glycoprotein encoded by ORF 2 (10, 11a). VP-2/M possesses three potential adjacent transmembrane segments close to the N-terminal end, and segments of only 9 to 13 amino acids are thought to extrude from the virion surface (see Fig. 4A). The primary envelope glycoprotein of EAV (G_L; 30 to 42 kDa) shown to be encoded by ORF 5 (255 amino acids) (10) may be the virus attachment protein, since neutralizing monoclonal antibodies (MAbs) are directed to this protein (26a). The primary envelope glycoprotein of LDV (VP-3; 25 to 42 kDa) (4, 5) is also encoded by ORF 5 (199 amino acids), and several different neutralizing MAbs specifically immunoprecipitate the ORF 5 protein (see below). The LDV ORF 5 protein has unusual properties for a principal viral envelope glycoprotein. It possesses a potential signal sequence at the N terminus and three potential transmembrane segments towards the N-terminal end (6, 14). After the removal of the signal peptide, only about 30 amino acids carrying one to three potential N-glycosylation sites may extrude from the virion surface (see Fig. 4B), an observation which might explain the smooth outer surface of the virions in electron microscopy (25). The minor ORF 2 envelope proteins of this group of viruses (227 to 256 amino acids) are more conventional viral envelope glycoproteins, possessing a signal peptide sequence, a transmembrane segment towards the C-terminal end, and one to three potential N-glycosylation sites in between (6, 9, 10, 14, 19).

The present study demonstrates that the nonglycosylated envelope protein (VP-2/M) and the VP-3 glycoprotein of LDV virions (strain P) (4) are covalently linked by disulfide bonds and that breakage of these bonds in virions correlates with loss of viral infectivity.

To examine the potential disulfide linkages between the structural proteins of LDV, ^{35}S-labeled proteins of LDV virions were analyzed by Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) (26) under reducing and nonreducing conditions. ^{35}S-amino-acid-labeled LDV was prepared by propagation in 1-day cultures of mouse peritoneal macrophages in 60-mm-diameter tissue culture dishes essentially as described previously (4, 5). The growth medium, RPMI supplemented with 10% (vol/vol) fetal bovine serum and 10% (vol/vol) L-cell-conditioned medium was replaced at 2 h postinoculation with RPMI lacking cysteine and methionine but containing 2% (vol/vol) fetal bovine serum. At 3 h postinoculation, the medium was supplemented with 200 μCi of Trans^{35}-LABEL (ICN, Costa Mesa, Calif.) per

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ml. The culture fluid harvested from two culture dishes at 20 h postinoculation was clarified by centrifugation at 10,000 $\times$ g for 10 min, and the virus was then collected by ultracentrifugation through a layer of 0.5 M sucrose in TNE (50 mM NaCl, 10 mM Tris-HCl, and 10 mM EDTA [pH 7.4]) in an SW41 rotor (Beckman ultracentrifuge) at 30,000 rpm for 5 h. The pelleted virus was lysed in 200 μl of phosphate lysis buffer (PLB) composed of 10 mM sodium phosphate (pH 7.5); 100 mM NaCl; 0.5% (wt/vol) sodium deoxycholate; 0.1% (wt/vol) SDS; 1% (vol/vol) Triton X-100; 0.5 mM phenylmethylsulfonyl fluoride; 1 μg each of aprotonin, leupeptin, and pepstatin A per ml; 0.25 mM iodoacetamide; and 20 mM N-ethylmaleimide. For immunoprecipitation, 30 μl of a lysate of ^{35}S-labeled LDV (2×10^6 cpm) was mixed with 345 μl of PLB and 75 μl of Protein G Sepharose 4 Fast Flow (Pharmacia, Piscataway, N.J.) (washed twice with PLB containing 5 mg of bovine serum albumin [BSA] per ml and twice with PLB containing 1 mg of BSA per ml and diluted 1:2 with the final wash buffer). The mixture was incubated with tumbling overnight at 4°C and centrifuged in an Eppendorf centrifuge. Samples of 30 μl of the supernatant fluid were incubated (with tumbling) with 3 μl of neutralizing MAb 159-19 (immunoglobulin G1) (15), B6506A7, or C7809A6 (immunoglobulin G3; kindly supplied by J.-P. Coutelier) (7, 8) at 4°C for 16 h. An equal volume of BSA-washed protein G-Sepharose was then added. The mixtures were further incubated with tumbling at room temperature (RT) for 4 h and centrifuged in an Eppendorf centrifuge. The pellet was washed five times in PLB and suspended in an equal volume of sample buffer consisting of 110 mM Tris-HCl, pH 6.8, containing 17% (vol/vol) glycerol, 5% (wt/vol) SDS, 0.35% bromphenol blue, and, only under reducing conditions, 8.4% (vol/vol) 2-mercaptoethanol. Nonimmunoprecipitated LDV was suspended in the same solutions. The suspensions were heated at 95°C for 10 min and clarified by centrifugation in an Eppendorf centrifuge. The supernatant fluids were analyzed by SDS-PAGE using Tricine buffer (27). The amounts of radioactivity in selected bands were estimated by scanning as described previously (17).

The first lane in Fig. 1A illustrates a typical SDS-PAGE profile of the three major structural proteins of LDV, VP-1/N, VP-2/M, and VP-3, analyzed under reducing conditions (4, 5). VP-3 migrates as a broad band between 25 and 42 kDa (4, 5). VP-3 consists mainly of a single protein that exhibits molecular size heterogeneity due to different degrees of glycosylation. This conclusion is indicated by the findings that treatment of the virion proteins with endoglycosidase F converted most of the 25- to 42-kDa protein band into a single protein migrating slightly faster than the VP-2/M protein (17 to 18 kDa) (11a) and that in Western blots (immunoblots) several anti-LDV MAbs react with the whole range of proteins in the 25- to 42-kDa band (7, 15). Other experiments, to be reported elsewhere (11a) confirm that this protein is encoded by ORF 5: in vitro transcription-translation of ORF 5 results in the production of a protein of about 19 kDa in the absence of, and a protein of about 28 kDa in the presence of, canine pancreatic microsomal membranes, indicating core glycosylation. Treatment with endoglycosidase F converts the 28-kDa protein to a 17- to 18-kDa protein just as it does VP-3 of LDV virions. This molecular mass is consistent with that of the ORF 5 protein after removal of the signal peptide (see Fig. 4B). Furthermore, the 27-kDa ORF 5 protein is precipitated by several VP-3-specific MAbs as well as by mouse antibodies to two peptides derived from ORF 5. The 25- to 42-kDa LDV protein band also seems to contain the ORF 2 glycoprotein of LDV, but it represents only a minor component (11a). For convenience we will continue to refer to the 25- to 42-kDa band as VP-3. Our

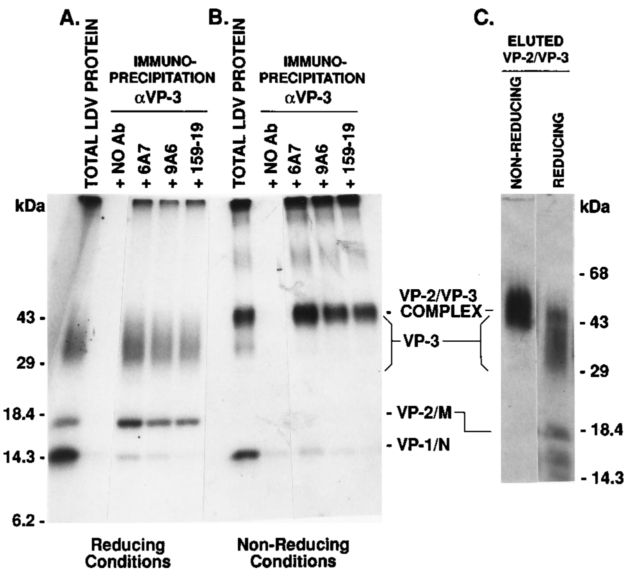

FIG. 1. Tricine-SDS-PAGE of ^{35}S-labeled LDV virion proteins under reducing (A) and nonreducing (B) conditions before and after immunoprecipitation by neutralizing anti (α)-LDV MAbs (see the text) and of an isolated VP-3–VP-2 dimer (C). 6A7 and 9A6, MAbs B6506A7 and C7809A6, respectively. For panel C, a lysate of ^{35}S-labeled virion proteins was electrophoresed under nonreducing conditions as for panel B, lane 1. The section of the gel containing the VP-2–VP-3 complex was excised, and the slice was cut vertically into two equal portions. The gel portions were incubated in sample buffer without (nonreducing conditions) or with (reducing conditions) 4.2% (vol/vol) 2-mercaptoethanol overnight and then analyzed by SDS-PAGE using Tricine buffer under reducing and nonreducing conditions, respectively.

results (11a) have also shown that ORF 6 is efficiently translated in vitro only in the presence of microsomal membranes. As expected, the about-19-kDa product is not affected by endoglycosidase F treatment.

Incubation of the ^{35}S-labeled LDV proteins with three neutralizing anti-VP-3 MAbs in the absence of reducing agents resulted in the precipitation of VP-3, but VP-2/M was also quantitatively precipitated (Fig. 1A). The coprecipitation of VP-2/M was unexpected since in Western blots these and other neutralizing MAbs react only with VP-3 (7, 15). These MAbs also do not precipitate the ORF 6 protein synthesized in vitro either when alone or when cotranslated in vitro with the ORF 5 protein (11a).

The reason for the coprecipitation of virion VP-3 and VP-2/M was resolved by electrophoresis of the immunoprecipitated LDV proteins under nonreducing conditions; all of VP-2/M comigrated with VP-3 in a complex of 45 to 55 kDa (Fig. 1B, first lane), and this complex was precipitated by all three neutralizing MAbs (Fig. 1B). Thus the results in Fig. 1A and B demonstrate that, in LDV virions, the VP-2/M and ORF 5 proteins are linked in a complex that is dissociated under reducing conditions that are known to break disulfide bonds. This conclusion was confirmed by cutting a slice containing the VP-2–VP-3 complex out of a gel, incubating equal portions in reducing or nonreducing buffers overnight, and then electrophoresing the proteins under reducing and nonreducing conditions, respectively (Fig. 1C). Both VP-3 and VP-2/M were released under the reducing conditions. The diffuse labeled proteins smaller than VP-2/M observed under reducing conditions probably represent degradation products generated during the overnight incubation of the gel slices under reducing conditions. LDV virions incubated overnight under reducing conditions show similar degradation products (data not

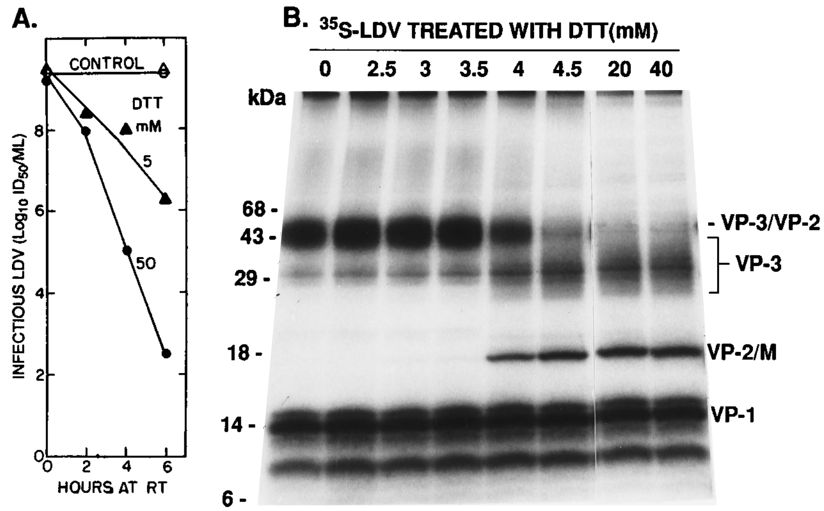

FIG. 2. Inactivation of LDV infectivity (A) and coincident dissociation of the VP-2–VP-3 complex of virions by incubation with DTT (B). For panel A, pooled plasma from 1-day LDV P-infected FVB mice (provided by the transgenic facility of the University of Minnesota) was clarified by centrifugation at 10,000 × *g* for 10 min, and LDV was collected from the supernatant by centrifugation through a layer of 0.5 M sucrose in TNE. The virus was suspended in balanced salt solution to the original volume of plasma. One portion of the LDV was incubated with 5 (▲) or 50 (●) mM DTT, and one portion was incubated without DTT (open symbols) for 6 h at RT. At the indicated times, 20-μl samples of virus suspension were removed, diluted 1,000-fold with balanced salt solution containing 3% (wt/vol) BSA, and analyzed for residual infectivity in mice by an end point dilution assay (23). Virus titers are expressed as 50% infectious doses (ID_{50}). Circles and triangles, results from two independent experiments. For panel B, samples of intact ^{35}S-labeled LDV were incubated with the indicated concentrations of DTT at RT for 2 h. The samples were mixed with sample buffer (without 2-mercaptoethanol [nonreducing conditions]), heated, and analyzed by SDS-PAGE using Tricine buffer.

shown). The two corresponding envelope proteins of EAV are probably similarly linked by disulfide bonds since an antibody to the EAV M protein coprecipitated the M and G_L proteins (10).

This is the first report demonstrating heteroduplex formation via disulfide bonds of a viral nonglycosylated, integral envelope protein and an integral envelope glycoprotein. The ratio of the ORF 5 protein and VP-2/M in the 45- to 55-kDa complex cannot be calculated with accuracy from our data but most likely is 1:1. The molecular weight of the VP3–VP-2/M complex is comparable to the sum of the molecular weights of the two proteins. The VP-3 band contained about three times more radioactivity than the VP-2/M band (Fig. 1A), but this radioactivity comparison is complicated by the fact that the proteins were labeled with both [^{35}S]cysteine (C) and [^{35}S]methionine (M) to achieve sufficient labeling and the amount of each incorporated into these proteins is unknown. The proteins also possess different numbers of C and M: five C and three M for VP-2/M and seven C and two M for the processed ORF 5 protein (see Fig. 4B). Furthermore, the minor ORF 2 protein (without signal peptide) in the VP-3 band possesses three C and nine M. Whether the latter is also disulfide linked cannot be deduced from the present data. The small amounts of about-80-kDa proteins apparent in nonreducing gels, which were precipitated by the anti-VP-3 MAbs (Fig. 1B), could represent heterodimers between the ORF 2 and ORF 5 proteins or ORF 5 protein homodimers. The labeled protein with a molecular mass of approximately 30 kDa in nonreducing gels (Fig. 1B, first lane) is not the ORF 2 protein but most likely a nonviral protein, since it is not precipitated by anti-VP-3 MAbs (Fig. 1B) or polyvalent antisera from LDV-infected mice (data not shown).

The results in Fig. 2A show that LDV infectivity was rapidly lost during 6 h of incubation with 5 mM dithiothreitol (DTT) at RT. Similar results were observed in repeated experiments. Incubation of LDV with 5 to 10 mM DTT for 6 h regularly resulted in a loss of ≥99% of its infectivity (see Fig. 3). The rate of inactivation was much higher with 50 mM than with 5 mM DTT (Fig. 2A). Little or no inactivation occurred during 6 h of incubation with 2.5 mM DTT (data not shown).

The results in Fig. 2B demonstrate that the inactivation of LDV infectivity by DTT correlated with dissociation of the VP-3–VP-2/M complex. When ^{35}S-labeled LDV was incubated for 2 h with DTT at a concentration of 3.5 mM or lower, with no effect on LDV infectivity, VP-3 and VP-2/M comigrated in the 45- to 55-kDa complex in a nonreducing gel (Fig. 2B). However, when the DTT concentration was increased to 4.0 to 4.5 mM, VP-3 and VP-2/M began to migrate as individual components in the nonreducing gel (Fig. 2B). Higher concentrations of DTT did not have any additional effects (Fig. 2B).

The inactivated LDV virions had about the same density (1.13 to 1.14 g/cm^3) (4, 25, 27) and about the same sedimentation coefficient (200 to 230S) (24, 25) as virions incubated for 6 h in the absence of DTT (Fig. 3). Repeated analyses showed that the slight difference in sedimentation rate between treated and control virions apparent in Fig. 3B was not significant. It represented a difference of less than one 0.3-ml fraction; similar differences were observed when untreated LDV was centrifuged in companion tubes, and the difference between treated and control virions has not been consistently observed. No disassembly of virions was associated with inactivation, because the amount of radioactivity recovered from the gradients containing inactivated virions was about the same as the amount recovered from gradients containing infectious virions (Fig. 3).

In summary, our results demonstrate that the two major envelope proteins of LDV virions, VP-2/M and the ORF 5 VP-3 protein, are linked by disulfide bonds and that these are essential for LDV infectivity. Disulfide bonds between the ectodomains of envelope glycoproteins are a common property of viral envelope glycoproteins and result in the formation of homo- or heterodimers, trimers, or tetramers. However, the situation for LDV virions is unusual in that the disulfide bonds are between a nonglycosylated integral envelope protein and

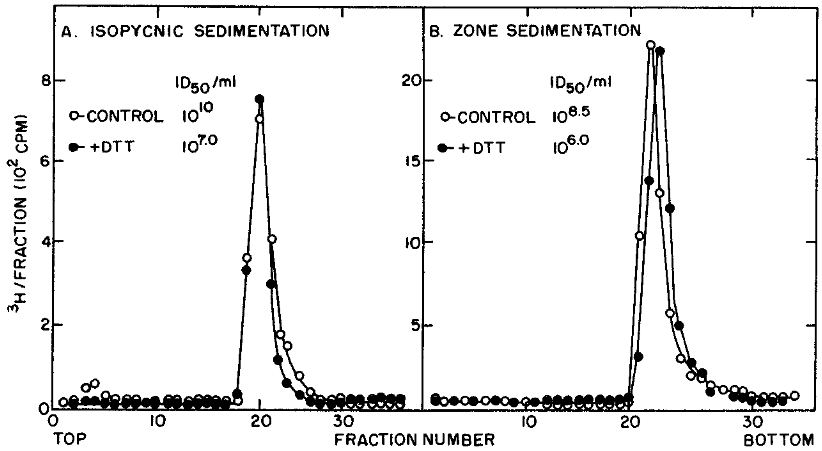

FIG. 3. Isopycnic (A) and zone (B) sedimentation analyses of ^{3}H-labeled LDV P after incubation in the absence (○) or presence (●) of DTT. [^{3}H]uridine-labeled LDV was prepared as described previously (4) and incubated with or without 10 mM DTT at RT for 6 h. Then, the samples were analyzed for infectious LDV and by centrifugation through gradients of 0.5 to 1.5 M (isopycnic sedimentation) or 0.15 to 0.9 M (zone sedimentation) sucrose in TNE in an SW41 rotor at 35,000 rpm for 4 h or 95 min, respectively. Fractions from the gradients were analyzed for ^{3}H in acid-insoluble material (27). The results in panels A and B were obtained with different pools of ^{3}H-labeled LDV.

an integral envelope glycoprotein and because the proposed ectodomains of these two proteins are very small (Fig. 4). The only situation that is somewhat similar is the formation of disulfide bonds between the 24-amino-acid N-terminal ectodomains of the M_2 protein of influenza virus to form homodimers that aggregate into an ion channel (16).

Furthermore, there are only a few cysteines that can participate in disulfide bond formation to generate VP-3–VP-2/M heterodimers. Two cysteines are located in the putative 30-amino-acid ectodomain of the ORF 5 VP-3 protein, two are located in the presumably internal C-terminal end of 74 amino acids very close to the third potential transmembrane segment, and three are located in the proposed transmembrane segments (Fig. 4B). The ORF 6 protein (VP-2/M) possesses one cysteine in the short N-terminal ectodomain, two in the internal C-terminal end, and two in the proposed middle transmembrane segment (Fig. 4A). It seems unlikely that cysteines in the postulated transmembrane segments or in the internal portions of the two proteins (Fig. 4) are involved in disulfide bonding. There does not seem to be any precedence for disulfide bond formation between membrane-spanning segments of viral proteins. Also, the internal, C-terminal portions of the virus proteins probably stay on the cytoplasmic side of the endoplasmic reticulum and are not exposed to the oxidizing environment and protein disulfide isomerase that facilitate disulfide bond formation in the lumens of endoplasmic reticulum vesicles (2, 3, 18).

It seems more probable that the disulfide bonds between the VP-3 and VP-2/M proteins involve their ectodomains, which are expected to be located in the lumens of endoplasmic reticulum vesicles. Most likely, C-1 of the ectodomain of VP-2/M and C-2 of the ectodomain of the ORF 5 protein are involved. This view is supported by the finding that these two cysteines are conserved in the proteins of both strains (C and P) of LDV (6, 14) and both strains (LV and VR2332) of porcine reproductive and respiratory syndrome virus (19, 21) that have been sequenced, as well as in the corresponding EAV proteins (9), even though, overall, the amino acids of their proteins diverge by 50 to 70% (6, 9, 14, 19, 21). C-1 is most likely the only cysteine of VP-2/M exposed on the virion surface.

Involvement of the ectodomains of VP-3 and VP-2/M in

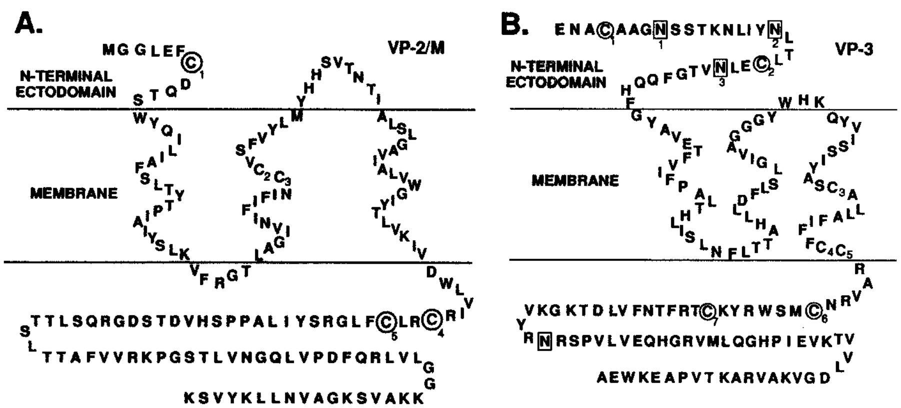

FIG. 4. Postulated membrane organizations of the ORF 6 (VP-2/M) and ORF 5 (VP-3) proteins of LDV P. Potential transmembrane segments have been deduced from hydrophobic moment analyses by the method of Eisenberg et al. (11; also, see reference 6). The mean hydrophobicities for the potential transmembrane segments starting from the N terminus are 49.2, 65.0, and 66.7%, for VP-2/M (A) and 54.1, 53.6, and 53.0% for VP-3 (B). The ORF 5 protein is shown after removal of the potential signal peptide (6). Cysteines in the ecto- and endodomains are circled, and potential N-glycosylation sites are boxed.

disulfide bonding is also most consistent with the finding that reduction of these bonds is not associated with an alteration in virion density or sedimentation rate. Virion structure may be maintained by the conformation of these proteins in the envelope which involves traversing the lipid bilayer several times (Fig. 4) and perhaps hydrophobic interactions between the proteins. One explanation for the loss of infectivity associated with the breakage of the disulfide bonds might be that the disulfide bonds between the ectodomains of VP-2/M and the ORF 5 VP-3 protein generate the surface site of the virion responsible for its binding to the LDV receptor on macrophages (25). Such arrangement does not contradict the finding that all neutralizing anti-LDV MAbs that have been examined are specific for the ORF 5 VP-3 protein (7, 11a, 15). However, it is still possible that the about-80-amino-acid C-terminal ends of VP-2/M and the ORF 5 VP-3 proteins are involved in disulfide bond formation. It is of interest that C-6 of the ORF 5 protein (Fig. 4B) is also conserved in LDV, porcine reproductive and respiratory syndrome virus, and EAV.

Overall, the results indicate that the envelope organization of LDV virions is markedly different from that of togavirus virions, even though superficially the morphologies of their virions are similar. In Sindbis virus, the envelope proteins form an icosahedral shell which is composed of trimers of heterodimers of the E1 and E2 spike proteins, with molecular masses of about 50 kDa (1, 12, 22). The outer shell is stabilized by disulfide bonds within the large ectodomains of the E1 and E2 proteins, which are rich in cysteine residues (13, 22). Disruption of the bonds by incubation with 5 mM DTT results in rapid loss of infectivity, just as observed for LDV, but in contrast to LDV, loss of infectivity is associated with disassembly of the envelope and release of the nucleocapsid (1, 12, 13). On the other hand, LDV virions seem much more unstable than Sindbis virus virions under other conditions not involving disulfide bond reduction (4, 20). For example, during preparation for electron microscopy the envelope of LDV virions tends to slough off, releasing cylindrical structures with a diameter of 8 to 14 nm (4). These structures may be composed of the VP-3–VP-2/M heterodimers. Furthermore, the envelope of LDV is removed from the nucleocapsid by treatment with Nonidet P-40 at concentrations as low as 0.01%, whereas at least 10-fold-higher Nonidet P-40 concentrations are required for removal of the envelope of Sindbis virus (4). These observations lead us to speculate that the primary function of the disulfide bonds between VP-3 and VP-2/M may be to stabilize the virus attachment site rather than to stabilize virion structure per se.

We thank Leslie A. Schiff and Mark E. Peeples for valuable advice for the immunoprecipitation and gel electrophoresis of LDV proteins and Colleen O'Neill for excellent secretarial help.

This work was supported by a grant from the Minnesota Medical Association and PHS training grant CA 09138 (K.S.F. and G.A.P.).

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