A Morphological Study of the M-Protein of Sendai Virus

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SUMMARY

A purification scheme is described for the M-protein of Sendai virus and an electron microscope study of the isolated protein is presented. The protein exists as subunits of 6 nm in diam., which possess a central hole; the subunits may be dimers of the polypeptide. They are able to form filamentous aggregates which wind around one another to form a helical structure. It is suggested that these filaments may be the form adopted by the protein in the virus, the filaments lying parallel to one another just beneath the virus membrane to form a shell, but that the helical form is likely to be a property only of the isolated protein.

INTRODUCTION

The M-protein (also known as the matrix, membrane or core protein) of the myxoviruses and paramyxoviruses is the smallest major protein of the virion. In the case of influenza virus it is believed to be located just beneath the membrane of the virus (Nermut, 1972; Schulze, 1972). Suggestions for the role of this class of proteins in the virus life cycle are that it serves as a recognition site for the attachment of nucleocapsid to the plasma membrane (McSharry et al. 1975; Shimizu & Ishida, 1975), that it organizes the envelope proteins of the virus (Blough & Tiffany, 1975) and that it is involved in the budding of the virus from the plasma membrane (Hewitt, 1976).

The M-protein of Sendai virus has a mol. wt., as assessed by SDS polyacrylamide electrophoresis, of 35,000. In this paper its purification is described; the procedure used is adapted from the work of Scheid & Choppin (1973) on the similar Newcastle disease virus. The morphological properties of the isolated protein are then described.

METHODS

General. All chemicals were of Analar grade or better. Operations were carried out at 0 to 5 °C unless otherwise specified.

Preparation of the virus. An inoculum of Sendai virus, kindly provided by the World Health Organization Influenza centre in this Institute, was diluted to approx. 0.01 haemagglutinating units/ml and 0.1 ml was injected into the allantoic cavity of 10-day-old embryonated eggs. The embryos were incubated at 35 °C for 3 days and stored overnight at 5 °C. The allantoic fluid was harvested and centrifuged at 500 g for 20 min. The pellet was discarded and the supernatant fluid centrifuged at 20,000 g for 2 h. The supernatant fluid was discarded and the pellet resuspended in a small volume (approx. 5 ml/litre of allantoic fluid) of phosphate buffered saline (PBS). The suspension of virus was then applied to a
gradient of 10 to 40 % (w/v) sucrose in PBS + 1 mM-EDTA and the whole was centrifuged at 15000 g for 30 min. The broad visible band of virus was removed with a syringe and the remainder of the gradient discarded. One hundred embryos yield 40 to 50 mg of virus as measured by protein assay (Lowry et al. 1951) using bovine serum albumin as a standard.

Preparation of the M-protein. The virus suspension was made 10^-4 M in phenyl methyl sulphonyl fluoride (PMSF) and dialysed overnight against 10 mM-potassium phosphate buffer, pH 7.2. The virus was then centrifuged at 20000 g for 1 h and the pellet resuspended in a sufficient volume of this buffer to give a protein concentration of approx. 5 mg/ml. One-tenth vol. of 20 % (v/v) Triton X-100 in 10 mM-potassium phosphate buffer, pH 7.2, was added and the sample stirred gently for about 15 min at room temperature. The sample was then centrifuged at 10000 g for 30 min and the supernatant fluid removed. This supernatant fraction contained most of the virus glycoproteins which could be further purified by centrifuging at 160000 g for 2 h, after which the pellet was discarded and the supernatant fluid designated fraction S.

The pellet from the 10000 g centrifugation, fraction P1, was resuspended in 10 mM-potassium phosphate buffer, pH 7.2, supplemented by 1 M-KCl and 2 % Triton X-100 and again centrifuged at 10000 g for 30 min. The pellet was designated fraction P2 and the supernatant fluid dialysed against 10 mM-potassium phosphate buffer, pH 7.2. A precipitate formed which consisted mainly of the M-protein; this was designated fraction M. The M-protein in this fraction is heavily contaminated with other virus proteins, particularly the nucleocapsid. To obtain a purer form of the M-protein the sample was redissolved in 1 M-KCl with 2 % Triton X-100 as above and the solution clarified by centrifuging at 20000 g; the supernatant fluid was then clear. The M-protein in the supernatant was again precipitated by dialysis into 10 mM-potassium phosphate buffer, pH 7.2, and these last steps repeated until a suitably pure preparation of the M-protein was obtained. This normally requires 4 to 5 cycles.

**SDS polyacrylamide gel electrophoresis.** The SDS-polyacrylamide gel electrophoresis used to monitor the preparation was essentially according to Laemmli (1970) using 7.5 % acrylamide and 0.3 % N,N-methylenebisacrylamide. The stacking gel described by Laemmli was omitted and the sample layered on to the gel with added glycerol for density stabilization.

**Electron microscopy.** For examination by negative staining a sample of fraction M was suspended in a low ionic strength buffer and washed with several changes of the buffer, with intermediate centrifugation, to remove detergent. The sample was then taken up in the appropriate medium. For the high ionic strength samples, a drop of sample was applied to a carbon-coated electron microscope grid and allowed to adsorb for 1 min. The grid was then washed on two successive drops of distilled water and stained. For low ionic strength samples a drop of sample was applied to a carbon-coated electron microscope grid, allowed to adsorb for 1 min, drained with filter paper and stained. Grids were examined in a Philips EM300 electron microscope.

Freeze-drying was carried out according to Nermut, Frank & Schäfer (1972) and thin sectioning after fixation according to Hirsch & Fedorko (1968).

Samples were inserted into the microscope upside down and the negatives printed with the emulsion side down in order to produce prints of the correct handedness.
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RESULTS

Chemical characterization of fraction M

SDS gel electrophoresis patterns of fractions M, S and P2 are shown in Fig. 1. For comparison a similar gel of whole Sendai virus is shown, labelled with the letter designations of the virus proteins according to Scheid & Choppin (1974). Fraction M can be seen to consist principally of the M-protein; the sample shown has been recycled through high and low salt concentrations four times. Scanning a gel of this fraction indicates that the Coomassie brilliant blue binding capacity of the protein in the nucleocapsid region of the gel is about 3% of that in the main M-protein band. The staining capacity at the dye front is somewhat variable with an average of about 10% of that in the M-protein band. The yield of M-protein is about 30% of the protein present in the input virus. The major loss of M-protein is of material remaining in the P2 fraction. Further extraction of this fraction with 1 M-KCl does not result in more M-protein being extracted into solution. The reason for this separation of the M-protein into two fractions is unclear.

Ultrastructural characterization of fraction M

Figure 2 shows structures seen after negative staining of fraction M. From a 1M-KCl solution most of the material is seen in the form of small aggregates with no discernible regularity (Fig. 2a). The apparent size of the subunits of which the aggregates are composed is about 6 ± 1 nm. Occasionally a central hole can be discerned in the subunit (Fig. 2b, arrowed). From a low salt suspension of fraction M tubular or cylindrical structures can be seen (Fig. 2c). The structure appears somewhat variable in size, varying from 13 to 20 nm in width. A close look at this structure reveals zig-zagging striations in the cylinder; the
striations themselves appear to have some subunit structure. This pattern is suggestive of a number of ribbons formed into a helix.

The use of the freeze-drying technique eliminates distortions of the specimen due to surface tension during the air-drying of conventional negative staining. In the present case it improves the regularity of the structure along its length (Fig. 3a) though the width of the
Fig. 3. Negatively stained, freeze-dried helices of M-protein. (a) Stained with phosphotungstic acid (pH 6·0); (b) high magnification picture, uranyl acetate stained. Subunits with an apparent central hole can be seen (arrows). (c) Uranyl acetate stained tube with filamentous form running from it.

tube is still variable. The greater tightness of the structure makes individual strands less easy to discern. Fig. 3(b) shows a high magnification picture of a uranyl acetate negatively stained, freeze-dried tube. Here, in places, the stain has imaged only one side of the helix and the strandedness of the structure is plainly visible. A central hole in the subunits of which the strands are composed can also be seen: this hole has a diam. of about 1·5 nm. Very
occasionally, structures can be seen in which some kind of filament is seen running off a tube (see Fig. 3c), suggestive of some filamentous substructure to the tubes.

Shadowing of a freeze-dried specimen visualizes only the upper surface of the structure; an example is shown in Fig. 4. This procedure shows up the helical stranded array particularly well. The pitch angle seen at the top of the helix is about 45°, the side by side spacing of the striations (that is the width of a strand measured perpendicularly to its length) is about 6 nm and the spacing along the length of the tube about 8.5 nm. The shadowed specimens establish that the helical array is left-handed. The apparent right-handedness of some of the negatively stained samples is a consequence of the two-sided image generated by this technique (see Finch & Klug, 1965).

The techniques used thus far in this article have the property of showing only that part of a sample which is able to adsorb to an electron microscope grid. Thin sectioning, on the other hand, samples input material randomly. For this reason a sample of fraction M, in a low ionic strength environment, was subjected to thin sectioning for electron microscopic examination. Material was seen to exist in the form of large aggregates, of no discernible structure, and, to a lesser degree, in filaments often running off the aggregates. These fila-
ments were of a size and form which appeared to correspond with the tubes seen in negative staining. The number of filaments was such as to make it unlikely that they arose from some contaminant other than M-protein.

Finally, mention must be made of the form adopted by any nucleocapsid which may contaminate fraction M in a low ionic strength solvent. This is shown in Fig. 5(a) while

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**Fig. 5.** Nucleocapsids negatively stained with phosphotungstic acid (pH 7.0). (a) Thickened form seen when the nucleocapsid is a contaminant of the M-protein preparation. (b) Normal form. This sample comes from fraction P2 of this paper.
Fig. 5(b) shows a normal nucleocapsid for comparison. It can be seen that the nucleocapsid in fraction M has a coat. Similar structures seen by Shimizu & Ishida (1975) and Yoshida et al. (1976) were interpreted as resulting from an association between the nucleocapsid and M-protein. Such a coating has also been seen on intracellular measles virus nucleocapsid (Almeida & Howatson, 1963).

DISCUSSION

The results presented here indicate that (1) the M-protein of Sendai virus exists as sub-units about 5 to 6 nm in diam. and which possess a central hole about 1.5 nm in diam. (2) The sub-units are able to aggregate to form filaments or ribbons which are stable at low ionic strength but are disrupted at high ionic strength. (3) The filaments may wind around one another to form a helical structure of the type illustrated in Fig. 6. In this diagram the number of strands in the helix is shown as three, the variability in the width of the tube makes this difficult to determine and it may be variable.

The mild conditions under which the protein was isolated encourage a belief that the form adopted by the isolated protein may be related to that of the protein in the virus. The features of the protein's ultrastructure which are dependant upon other virus components will not be observed of course, but those features of the protein's arrangement in the virus, which results from its self-affinity, might be reflected in the structure formed by the isolated protein. This would suggest that in the virus the M-protein may exist as filaments, arranged, by analogy with influenza virus, in such a way as to form a shell just beneath the membrane of the virus. Support for a filamentous organization of the M-protein of influenza virus comes from the work of Nermut (1972) and Schulze (1972). These workers studied influenza virus cores, i.e. particles from which the virus membrane has been removed. On the surface of a small proportion of these cores, marked parallel striations could be seen, rather reminiscent of the filaments seen in the present study. This suggests that these striations reflect the form of the M-protein in the virus. Unfortunately, similar results have not, so far, been obtained with a paramyxovirus, though the transcapsidation of the two classes (Granoff & Hirst, 1954) suggests that the two membranes are organized on similar principles.

Further support for the structure of the M-protein described here being related to that adopted in the virus comes from the form of contaminating nucleocapsid in the M-protein preparation. The important point here is that each virus component must have specific affinity for at least one other virus component in order to be incorporated into the virion and there is evidence that the M-protein and the nucleocapsid have specific affinity for one another (Shimizu & Ishida, 1975); that is, binding to nucleocapsid is one of the biochemical functions of the M-protein. The M-protein prepared as described here appears to have retained this ability; thus it is, by this criterion, functionally native. This implies that the structure adopted by the M-protein will be relevant.

The helical conformation adopted by the protein in a low ionic strength environment, is
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most likely to be merely a conformation adopted by the isolated protein and does not reflect the arrangement of the protein in the virus. It should be noted, however, that this structure, and the arrangement in the virus suggested here from the work of Nermut (1972) and Schulze (1972), are both parallel arrangements of filaments. Thus the M-protein to M-protein contacts can be the same in both structures. The helical form seems to be related to the thickened nucleocapsid, but there is no evidence from the intact virus of the nucleocapsid possessing a coating of M-protein. It seems best, therefore, to consider that the helical form arises from a quaternary rearrangement of the protein after isolation. Reference should be made at this point to the work of McSharry et al. (1975). These workers examined the structure of the isolated M-protein of paramyxovirus SV5; no detailed structure was visible, though the material had a fibrous appearance. The absence of internal structure may be due to the different virus type or to the more stringent conditions used in isolating the protein.

A crude model for the M-protein subunit is a disc, about 6 nm in diam. with a central hole about 1.5 nm in diam. The height of the disc is about 3.5 nm, since the increase in thickness of nucleocapsid when it acquires a coat of M-protein is about 7 nm. From the relationship between size and mol. wt. discussed by Green (1969), this model would suggest a mol. wt. for the subunit of about 65000, suggesting that subunits may be dimers of the individual M-protein polypeptide. The existence of the central hole would be unusual if the subunit were a monomer; this may also suggest that the subunit is some higher polymer of the polypeptide. Electron microscope data alone, however, cannot be conclusive on the degree of aggregation.

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REFERENCES


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