Controlled Proteolytic Digestion of the M-protein of Sendai Virus: the Isolation of a Fragment of 30000 Molecular Weight

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SUMMARY

Proteolytic digestion of the M-protein of Sendai virus produces a product with a mol. wt. approximately 5000 less than that of the intact protein. In the case of digestion with chymotrypsin this cleavage is quite specific and the cleaved protein can be isolated. The smaller fragment appears to be physically removed from the larger (30000 mol. wt.) fragment, rather than remaining in non-covalent association with it. The cleavage is likely to be near the N-terminus of the protein. At the present time there is no indication of the biological function of this fragment.

INTRODUCTION

During the course of a study of the isolated M-protein of Sendai virus (Hewitt & Nermut, 1977) it was noticed that it was necessary to inhibit any endogenous proteases using phenyl methyl sulphonyl fluoride (PMSF). Omission of this step resulted in substantial degradation of the protein. A major product of the degradation was found to be a fragment which co-purified with the intact protein and possessed a mol. wt. of 30000 as assayed by SDS polyacrylamide gel electrophoresis, compared with the intact protein which has a mol. wt. of 35000 by the same criterion. It seemed to us worthwhile to pursue the characterization of this cleavage further, with a view to obtaining information about the nature of this fragment in the protein's structure, and ultimately on any separation of function within the molecule which this cleavage might reflect. We describe here the induction of this cleavage using pure exogenous enzymes, the isolation of the major product and its protein chemical characterization.

METHODS

Materials. PMSF, chymotrypsin (EC 3.4.4.5) and trypsin (EC 3.4.4.4) were from Sigma Chemical Co. (St Louis, U.S.A.). Sendai virus and its M-protein were prepared according to Hewitt & Nermut (1977). The cleaved form of the protein, here designated fraction M', was prepared by making a sample of virus 10^-4 M in PMSF, to inhibit any endogenous protease, and dialysing into 10 mm-potassium phosphate buffer of pH 7.2. Three μg/ml of chymotrypsin were then added to the virus preparation and the whole made 2% (v/v) in Triton X-100 by addition of 1/10 volume of a 20% (v/v) solution of Triton X-100 in the same buffer. This mixture was stirred gently for 1 h at room temperature and the mixture then

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made 10^{-4}M in PMSF and centrifuged for 30 min at 10000 g and 5^°C. The pellet was resuspended in 10 mm-potassium phosphate buffer, pH 7.2, containing 2% Triton X-100 and 10^{-4}M-PMSF and again centrifuged at 10000 g for 30 min at 5^°C. The pellet was then extracted with 1 m-potassium chloride, 10 mm-potassium phosphate buffer, pH 7.2, 2% Triton X-100 and suspension centrifuged at 20000 g for 1 h. The M'-protein in the supernatant was then purified by repeated cycling through high and low ionic strength as described by Hewitt & Nermut (1977) for the intact protein.

Methods. Electron microscopy was carried out as described by Hewitt & Nermut (1977). Polyacrylamide gel electrophoresis was also done according to these workers except that 10% acrylamide, 0.3% N,N-methylenebisacrylamide was used.

Protein chemistry

Reduction and carboxymethylation. Samples of M-protein and M'-protein were dissolved at concentrations of 0.7 mg/ml in a solution containing 6 M-guanidinium chloride, 50 mm-tris/HCl, pH 8.4, 1 mm-EDTA and 1 mm-dithiothreitol. After 1.5 h at 20 °C, 2-14C-iodoacetic acid (the Radiochemical Centre, Amersham, U.K.), with a specific activity of 1500 ct/min/nmol, was added to a concentration of 4 mm. The solutions were incubated in the dark under N_2 at 20 °C for 2 h, then made 10 mM in dithiothreitol and dialysed exhaustively against 50 mM-NH_4HCO_3 and freeze-dried.

Amino acid analyses. Samples of reduced carboxymethylated protein, and performic acid oxidized protein (by the method of Hirs, 1967) were hydrolysed in vacuo at 110 °C for 22 or 70 h in 6 M-HCl containing 0.1% (w/v) phenol. The acid was removed in vacuo over NaOH, and amino acid analyses were performed on a Biocal Analyzer, No. BC.100. Tryptophan was estimated by the methanesulphonic acid hydrolysis method of Liu (referred to in Moore, 1972), after correction for 40% destruction during hydrolysis (estimated by comparison with known protein). For the intact M-protein, tryptophan was also estimated by the spectral method of Edelhoch (1967), using 6 m-guanidinium chloride as the solvent.

N-terminus determination. The dansyl method for proteins (Gray, 1972) was applied to samples (approx. 1 nmol) of M and M', both unmodified and reduced and carboxymethylated. Dansyl amino acids were identified by chromatography on micropolyamide layers (Bruton & Hartley, 1970).

C-terminus determination. Samples of reduced, carboxymethylated M and M' were treated with carboxypeptidase A (EC 3.4.2.1) and a mixture of carboxypeptidase A and carboxypeptidase B (EC 3.4.2.2), essentially as described by Guidotti (1960). The small quantities of material available would make the quantitative assay essential for unambiguous determination of the C-terminal residues very expensive. A qualitative assay for liberated amino acids was performed by dansylation, followed by chromatography on micropolyamide layers (Bruton & Hartley, 1970).

Sequence analysis. The method of Weiner et al. (1972) was applied to samples of reduced carboxymethylated M and M'.
Peptide maps. Fifteen μg samples of reduced, 14C-carboxymethylated M and M' were suspended in 0.1 M-NH4HCO₃ (30 μl). Trypsin (0.5 μg in 5 μl H₂O of Worthington lot No. TRB-3BB) was added to each and the samples incubated, with occasional shaking, at 37 °C for 1 h. The material was freeze-dried, the digests taken up in pH 6.5 buffer (pyridine-acetic acid-water, 20:1:189 by volume) and applied to cellulose thin layer plates (20 × 10 cm). The plates were wetted with pH 6.5 buffer and electrophoresis performed at 20 V/cm for 1 h at 15 °C in the long direction. The plates were dried in warm air and chromatography was performed perpendicularly in BAWP (butanol-acetic acid-water-pyridine, 15:3:12:10 by volume). The dried plates were stained for arginine containing peptides with phenanthrenequinone-NaOH reagent (Yamada & Hans, 1966), or stained with cadmium ninhydrin reagent and autoradiographed.

RESULTS

Fig. 1 shows the results of digestion of the M-protein with enzymes. Fig. 1(a) and (b) show the digestion with trypsin in low and high ionic strength conditions respectively. A 30,000 fragment is produced but the digestion rapidly proceeds to low mol. wt. products. The banding patterns produced in low and high ionic strengths by digestion with trypsin are only slightly different, though the rates of digestion are increased by the dissolution of the M-protein in the high ionic strength solvent. The digestion of the M-protein with chymotrypsin (Fig. 1c) also produces a 30,000 mol. wt. fragment, but in this case the progress of the digestion to lower mol. wt. fragments is slower. This enzyme was therefore selected for attempts to isolate the major fragment. The result of such isolation is shown in Fig. 1(d); here samples of intact virus, isolated M-protein, and fraction M', prepared as in Methods, are shown for comparison. It can be seen that fraction M' consists predominantly of the major, 30,000 mol. wt., fragment. It is on samples prepared in this way using chymotrypsin digestion that protein chemical studies were performed.

Amino acid analyses of M-protein and M' are presented in Table 1. The results are the averages of several analyses (6 for M-protein and 5 for M'-protein). Serine and threonine were corrected for destruction during hydrolysis, by extrapolation to zero time from the results obtained after 22 and 70 h of hydrolysis. The data for valine and isoleucine were those obtained after 70 h of hydrolysis. The results are expressed as the number of residues per polypeptide chain, based on mol. wt. of 35,000 and 30,000 for M and M' respectively.

No N-terminal residue was detected in M-protein, and no α-dansylated amino acid was detected during three cycles of dansyl Edman degradation. Investigation of the 30,000 fragment derived by chymotryptic digestion revealed an N-terminus of arginine. The second and third residues were determined by dansyl Edman degradation to be threonine and glycine respectively.

The results of the C-terminal investigation were similar for M and M', with leucine as the only residue liberated in significant amounts by carboxypeptidase A digestion. The inclusion of carboxypeptidase B produced no further digestion products.

Staining of the tryptic peptide maps with ninhydrin did not reveal any major differences between M and M' (see Fig. 2a). Several minor differences (the spots corresponding to spots 4, 5 and 6 in the diagrammatic map, Fig. 2d) might be interpreted as reflecting the cleavage in the M-protein. Staining of the peptide maps with the arginine specific phenanthrenequinone-NaOH reagent reproducibly revealed the presence of three acidic arginine-containing peptides in M but not in M' (see Fig. 2b, the spots marked 1, 2, and 3 in the diagrammatic map). One of these spots may be the N-terminus of the protein, which, if
Fig. 1. SDS polyacrylamide gels displaying digestion of M-protein with enzymes. (a, b) Digestion of M-protein with trypsin in low and high ionic strength respectively. Digestion conditions were approx. 1 mg/ml protein, 2 μg/ml trypsin, 37 °C in 0.01 M-potassium phosphate buffer, pH 7.2, 2 % Triton X-100; (b) contains 1 M-NaCl. The figures on the gels indicate digestion time in minutes. (c) Digestion with 3 μg/ml chymotrypsin; other conditions were the same as in (b). (d) Fraction M' with whole virus and intact M-protein shown for comparison.
Table 1. The amino acid compositions of purified specimens of M-protein and M'-protein and the difference between them*

<table>
<thead>
<tr>
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<th>M</th>
<th>M'</th>
<th>Δ</th>
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<tr>
<td>Asp</td>
<td>29.3 ± 1.1</td>
<td>23.4 ± 2.0</td>
<td>5.9</td>
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<tr>
<td>Thr</td>
<td>20.6 ± 0.8</td>
<td>19.0 ± 0.5</td>
<td>1.6</td>
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<tr>
<td>Ser</td>
<td>20.0 ± 1.5</td>
<td>19.1 ± 0.5</td>
<td>0.9</td>
</tr>
<tr>
<td>Glu</td>
<td>21.9 ± 1.6</td>
<td>19.0 ± 0.5</td>
<td>2.9</td>
</tr>
<tr>
<td>Pro</td>
<td>19.4 ± 1.6</td>
<td>15.9 ± 1.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Gly</td>
<td>28.8 ± 2.0</td>
<td>25.0 ± 1.0</td>
<td>3.8</td>
</tr>
<tr>
<td>Ala</td>
<td>20.5 ± 1.3</td>
<td>17.8 ± 0.5</td>
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<tr>
<td>ßCys</td>
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<td>4.3 ± 0.3</td>
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<tr>
<td>Val</td>
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<tr>
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<td>Leu</td>
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<td>Lys</td>
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<td>3.7</td>
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<td>His</td>
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<tr>
<td>Arg</td>
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<td>15.4 ± 0.3</td>
<td>3.0</td>
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<tr>
<td>Trp</td>
<td>31.1 ± 1.0</td>
<td>24.1 ± 1.0</td>
<td>0.7</td>
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</table>

* The compositions are calculated on the basis of mol. wt. of 35000 and 30000 for M and M' respectively. Cysteine was determined as cysteic acid and methionine as methionine sulphone. The ranges of results from 6 analyses for M-protein and 5 analyses for M'-protein are given.

Discussion

The proteases trypsin and chymotrypsin are able to cleave the M-protein to produce a product with a mol. wt. of 5000 less than the intact protein. In the case of chymotrypsin the cleavage is relatively specific and a species can be isolated consisting predominantly of the cleaved form. The N-terminal sequencing experiments indicate that a new N-terminus is generated by the cleavage. The carboxypeptidase digestion experiments do not show that any new C-terminus is generated, though, to the extent that chymotrypsin can cleave at leucine, we cannot exclude the possibility that a new C-terminus of leucine is generated. These observations are most easily described by a cleavage 5000 daltons from the N-terminus of the protein. The smaller fragment appears to be physically removed from the protein; the evidence for this comes from (1) the absence of a new C-terminus; this experiment was not...
Fig. 2. Tryptic peptide maps of fractions M and M'. Electrophoresis at pH 6.5 was followed by chromatography in BAWP on cellulose thin layers. The maps appear as mirror images, the upper being M and the lower M' in each case. (a) Photograph of maps stained with ninhydrin – Cd reagent (b) Fluorogram of maps stained for arginine with phanethrenequinone-NaOH reagent. (c) Autoradiograph of peptide maps. In this experiment less M'-protein is present than M-protein. (d) Schematic description of peptide maps. Key: Spots surrounded by a line, ninhydrin-Cd staining spots; Y, yellow colour with ninhydrin-Cd; Y → P, initial yellow colour with ninhydrin-Cd, becoming pink; cross-hatching, \( / \), \( ^{14} \)C-carboxymethyl cysteine containing peptides (autoradiograph); cross-hatching \( // / \), arginine containing peptides (phenanthrenequinone stain); solid spots, carboxymethyl-cysteine and arginine containing peptides. X, xylene cyanol FF, dye marker; \( e \), \( e \)-dinitrophenyl-lysine marker; 1, 2, 3, 6, peptides in M absent from M'; 4, peptide in M staining more intensely than in M'; 5, 5', different peptides present in M and M'; Arg, position of arginine after pH 6.5 electrophoresis.
quantitative and the possibility of two C-termini of leucine is not excluded. (2) The peptide map of the cleaved protein contains less spots than that of the intact protein. It is possible to describe all these data in terms of two cleavages, one at either end of the protein, but we feel that this is unlikely.
The electron microscopic appearance of the cleaved form is virtually identical with that of the intact protein. The cleaved protein will form helices and will bind to the nucleocapsid. These observations suggest that the 5000 mol. wt. fragment is not located at those sites of the protein which are involved in helix formation or nucleocapsid binding. They also suggest that the fragment is not located in the main body of the protein but is an appendix to it, thus allowing its removal without any gross conformational changes in the protein.

The trypsin digestion patterns appear essentially the same in high and low ionic strength conditions, although the rate of digestion changes. This suggests that the protein does not undergo any gross conformational change upon dissolution in a high ionic strength solution. The rate change is likely to be a reflection of the change in the physical state of the protein from precipitate to solution.

Hewitt & Nermut (1977) suggested that the M-protein exists as a dimer of its polypeptide, and there is now physicochemical evidence in support of this suggestion (Hewitt, 1977). These studies leave us with a picture of the M-protein subunit as a dimeric disc-like entity with a central hole; one side of the disc is able to interact with nucleocapsid. The most likely location for the cleavable fragment is on the opposite face of the disc to the nucleocapsid binding site. This would account for the failure of its removal to influence coated nucleocapsid formation or the side to side interaction seen in helix formation. This side of the disc should be the membrane side.

It has been suggested (Yoshida et al. 1976, and other workers) that the M-protein acts as a glue between the spike proteins/membrane and the nucleocapsid of the virus. This scheme, or a related one is almost certainly necessary to account for the specificity of virus assembly. On this basis one might reasonably speculate that the 5000 mol. wt. fragment could be involved in the binding of the M-protein to the membrane, the association between the M-protein and the spike protein, or both.

The involvement of a fragment of 5000 mol. wt. has been reported in the binding of several membrane proteins to the membrane (Brand & Skehel, 1972; Spatz & Strittmatter, 1971; Utermann & Simons, 1974; Segrest et al. 1972). The question arises whether this fragment is functioning in a like manner. The data presented here contain few indications on this topic; the amino acid analyses do not indicate that the fragment is heavily enriched in hydrophobic amino acids; one would expect that a membrane binding fragment would be. Experiments in this laboratory to test directly the possibility that this fragment is of the membrane binding type are, so far, incomplete. They tend, however, to suggest that the 5000 mol. wt. fragment may not be uniquely involved in binding the M-protein to the membrane. If substantiated by further work, such a conclusion will make it worthwhile to test the possibility that this fragment is involved in an association between the M-protein and the spike proteins of the virus.

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REFERENCES


M-protein cleavage


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