Binding of Tobacco Mosaic Virus to Membrane Material Isolated from Tobacco Leaves

Yukio KIHO and Toru SHIMOMURA

Institute for Plant Virus Research, Chiba

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ABSTRACT

Binding of tobacco mosaic virus (TMV) to disrupted tobacco leaf membrane was studied. Membrane isolated from tobacco leaves was treated successively with (NH₄)₂SO₄, Li-diodosalicylate and then pronase. TMV-binding substance was thus isolated in a soluble form. From enzymatic digestion experiments, it was suggested that the binding substance was composed of lipid and carbohydrate.

Polyornithine-induced adsorption of tobacco mosaic virus (TMV) to membrane isolated from tobacco leaves was reported [2]. Several pieces of evidence suggested that TMV was bound directly to the membrane and polyornithine stimulated this process by strengthening ionic binding between TMV and the membrane (unpublished data). In the work reported herein, tobacco leaf membrane was disrupted by a detergent, Li-diodosalicylate, and fractionated to obtain a substance involved in the binding of TMV to membrane. Here, we assume substance R as a membrane component which combines directly with TMV.

MATERIALS AND METHODS

Tobacco plant (Nicotiana tabacum var. Xanthi) and OM strain of TMV labeled with ¹⁴C-amino acids (¹⁴C-TMV, 4 × 10⁶ cpm/mg TMV) were used. Methods for fractionation of leaf extract and preparation of membrane by sucrose gradient centrifugation were reported previously [2]. Disruption and further fractionation of the membrane (P₁B fraction in previous report [2]) were done as shown in Figure 1. Membrane washed with 1 M (NH₄)₂SO₄ was disrupted by 0.3 M lithium 3,5-diodosalicylate (LIS). LIS was first used for preparation of glycoprotein from mammalian cell membrane by Marchesi and Andrews [4]. The relative amounts of protein released from the membrane into the supernatant after a low speed centrifugation (10 000 × g, 5 min) were about

Membrane fraction (60 mg protein in 4 ml Tris-HCl buffer, 0.01 M, pH 7.4)
↓
Washed with 4 ml of 2 M (NH₄)₂SO₄ solution
↓
Resuspended in 2.4 ml Tris-HCl buffer containing 0.3 M LIS
↓
Vigorously shaken for 15 min
↓
Mixed with 5 ml Tris-HCl buffer and shaken for 15 min
↓
Dialyzed against water for 24 hr at 4°C (preparation A)
↓
Digested with pronase (1.2 mg) at 37°C for 2 hr in 0.01 M Tris-HCl buffer
↓
Supernatant after 10 000 × g, 10-min centrifugation
↓
Kept overnight in a refrigerator
↓
Again centrifuged at 10 000 × g, 10 min
↓
Supernatant (preparation B)

Fig. 1. Fractionation of membrane.
5% and 40 to 50% by the (NH₄)₂SO₄ washing and LIS treatment, respectively. After dialysis against water to remove free and dissociated LIS, preparation A was obtained. Preparation A was then treated with pronase in Tris-HCl buffer, pH 7.4 at 37°C for 2 hr. The amount of pronase used was one-fiftieth of the protein content in preparation A. Materials released by pronase digestion were obtained in the supernatant by a low speed centrifugation (preparation B).

Binding of TMV to the membrane materials was assayed in the presence or absence of Ca++. Preparation A or B (1 mg protein content or its equivalent derived from preparation A) was mixed with ¹⁴C-TMV (1000 cpm, 100 μg) in 1 ml of 0.1 M acetate buffer, pH 5.0, containing 0.05 M Ca(NO₃)₂ (with addition of Ca at the end). After an incubation at 25°C for 0.5-3 hr, the mixture was centrifuged at 10,000×g for 5 min, and radioactivities in the supernatant and precipitate were measured by a gas flow counter (Aloka, PDC-303) to determine free and bound TMV. It was found that TMV bound in the presence of Ca++ was not released by 1 M NaCl. Hereafter, this binding will be designated as Ca-dependent binding. In the presence of Ca++.

Experiment 1 of Table 1 shows the binding activity (in the absence of Ca++) of preparation A. Thirty-five per cent of the input ¹⁴C-TMV was bound. A similar binding was observed with undisrupted original membrane, polyornithine being required for efficient binding [2]. When preparation A was digested with pronase (pronased A), very little ¹⁴C-TMV was observed in the precipitates. Since no ¹⁴C released from ¹⁴C-amino acid-labeled TMV by pronase, membrane protein should be involved in the TMV binding. As to the function of this protein, two possibilities may be considered: 1) Binding substance R is a protein. Therefore TMV cannot bind to pronased A. 2) Alternatively, binding substance R exists as a complex with protein embedded in the membrane. Digestion of preparation A by pronase leads to release of binding substance R from the membrane. Although R-TMV complex may be formed, this complex can not precipitate by the low speed centrifugation. The following experiments supported the second possibility.

Preparation B (materials released by pronase from the (NH₄)₂SO₄ and LIS-treated membrane) had the ability to suppress the infectivity, as shown in Table 2. When preparation B was applied on leaves already inoculated with TMV, inhibition occurred to a lesser extent than when preparation B was applied before TMV inoculation. The simplest explanation of these results is that substance R inactivated TMV.

Table 1. Binding activities of disrupted membrane

| Expt. number | Cpm in precipitate (bound TMV) |
|--------------|-------------------|-------------------|
| Preparation  | without Ca++      | with Ca++         |
| 1            |                   |                   |
| A            | 351               | 70                |
| Pronased A   |                   |                   |
| 2            |                   |                   |
| A            | 183               | 906               |
| Pronased A   |                   |                   |
| B            | 841               |                   |

¹⁴C-TMV (1000 cpm) was added to disrupted membrane (preparation A or B). Bound TMV was measured after centrifugation. See Materials and Methods for details.
by combining with the latter. Preparation B contained pronase. However, a control experiment showed that pronase had no effect on the TMV infectivity under the conditions of our experiments.

b) Experiment 2 of Table 1 shows the Ca-dependent binding activities of preparation A, pronased A and B. In contrast to the binding in the absence of Ca++ , the Ca-dependent binding activity of preparation A was enhanced after pronase treatment. Furthermore, a similar efficient Ca-dependent binding was also observed with preparation B. Preparation B alone was found to precipitate in the presence of Ca++. However, TMV was not bound to this aggregates. Thus the Ca-dependent binding reaction seemed to proceed in the following way. TMV was first bound to R. Ca++ was not required in this step and R-TMV was still soluble. Upon addition of Ca++, R-TMV complex became aggregated and could be precipitated by the low speed centrifugation.

Some control experiments were performed. No Ca-dependent binding activity could be demonstrated without membrane materials or without Ca++. LIS at a high concentration (higher than $5 \times 10^{-5}$ M) produced white precipitates with TMV in the presence of Ca++ at pH 5.0. However, as the amount of LIS remaining in preparation B was less than $5 \times 10^{-5}$ M (deduced from the optical density at 320 nm for LIS), the binding activity could not be ascribed solely to the action of the residual LIS.

At present, a feasible interpretation of the results of Table 1 is that the binding substance R exists inside the membrane as a complex with protein (protein-R). Due to the presence of protein which covers substance R in some way or other, the attachment of TMV to substance R is not very easy (Table 1; Expt. 1, A or Expt. 2, A). By pronase digestion, R is released from the membrane. Binding between TMV and free R, then, readily takes place. Although the R-TMV complex is too small to be precipitated by a low speed centrifugation (Table 1; Expt. 1, pronase A), viral infectivity is suppressed in this condition (Table 2). Upon addition of Ca++, the R-TMV complex became aggregated and precipitated by the low speed centrifugation (Table 1; Expt. 2, pronased A or B).

Reaction Time and Temperature
In the case of intact membrane, the Ca-

Table 2. Suppression of TMV infectivity by preparation B

<table>
<thead>
<tr>
<th>Expt. number</th>
<th>Inoculation condition</th>
<th>Average number of local lesions</th>
<th>% inhibition of infectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TMV</td>
<td>143</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>TMV + preparation B</td>
<td>15</td>
<td>89</td>
</tr>
<tr>
<td>2</td>
<td>B, 6 hr before TMV</td>
<td>304</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>B, 6 hr after TMV</td>
<td>37</td>
<td>88</td>
</tr>
</tbody>
</table>

Expt. 1: TMV with or without preparation B was inoculated onto tobacco leaves (Samson NN).
Expt. 2: Preparation B was applied on tobacco leaves 6 hr before or after TMV inoculation.
Preparation B: 0.2 mg protein equivalent was given per 2.5 µg TMV.

Table 3. Kinetics of the binding reaction

<table>
<thead>
<tr>
<th>Expt. number</th>
<th>Preparation</th>
<th>Reaction time (hr)</th>
<th>Reaction temperature (C)</th>
<th>Ca-dependent binding (% bound)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>0.5</td>
<td>30</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>2</td>
<td>30</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>4</td>
<td>30</td>
<td>74</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
<td>1</td>
<td>30</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>3.5</td>
<td>30</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.5</td>
<td>0</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.5</td>
<td>30</td>
<td>63</td>
</tr>
</tbody>
</table>

Binding of 14C-TMV to disrupted membrane (preparation A or B) was measured under various conditions. See Materials and Methods for details.
dependent binding requires a prolonged incubation at 30°C [2]. Similar requirements were also observed with preparation A (Table 3; Expt. 1). In the case of preparation B, however, an efficient binding took place rapidly even at a low temperature (Expt. 2). Thus, if the binding substance R was released from the membrane by pronase digestion, TMV was bound to R very easily.

*Nature of the Binding Substance R*

When preparation B was treated with Triton X-100 (0.5% at a final concentration), the binding activity was almost completely lost. Also, bound TMV formed in the presence of Ca++ was dissociated by Triton X-100 and more than 80% of the bound TMV was released by this treatment.

As a model experiment, several phospholipids were used to see whether they had a similar binding activity or not. To a reaction mixture heretofore used for determination of the Ca-dependent binding activity, were added phospholipids (0.5–5 mg) instead of preparation B. Results are shown in Table 4. With lecithin and cephalin, no TMV precipitation was observed, although cephalin was precipitated under this condition. Sphingomyelin, at a fairly high concentration, precipitated TMV. Further interpretation of this experiment, however, should await identification of the binding substance R.

Effect of lipase and glycosidase (mixture of various glycosidases) on substance R was investigated. Preparation B (1 mg protein equivalent) was treated with lipase or glycosidase at various concentrations in 0.01 M Tris-HCl buffer pH 7.4 at 37°C for 1 hr. Ca-dependent binding of TMV to these digested samples were determined.

**DISCUSSION**

TMV-binding substance R was isolated.
from membrane. By successive treatments with (NH₄)₂SO₄, LIS and pronase, R could be released in a soluble form. Single treatment with (NH₄)₂SO₄, LIS or pronase was not effective. (NH₄)₂SO₄ can be replaced by KCl. At least a part of ionic bonds in the membrane could be cleaved by a high concentration of these salt. Washing of membrane with buffer of a low ionic strength after (NH₄)₂SO₄ treatment (before LIS treatment), was not effective, probably because some ionic bonds could be restored during the washing with buffer. Simulta-neous addition of (NH₄)₂SO₄ and LIS to the membrane preparation was also not suitable. When (NH₄)₂SO₄ was added to an LIS solution, a white precipitate was formed, resulting in a decrease in the effective amount of LIS. For this reason, LIS was added to the membrane preparation after (NH₄)₂SO₄ treatment of the latter. These facts suggest that R is interior to the membrane and is surrounded by many bonds of various types.

As reported previously [2], the Ca-dependent binding of TMV to membrane requires a certain temperature and prolonged incubation. Furthermore, polyornithine was necessary for an efficient binding. In contrast to the membrane, the reaction between TMV and the isolated R takes place very easily and requires no polyornithine. As this remarkable change is observed after pronase digestion of disrupted membrane, it is possible that protein masks the binding substance R inside the membrane, not rendering it easily accessible to TMV. In the case of polyornithine-stimulated adsorption of TMV to membrane, it is possible that polyornithine might combine with the above-mentioned protein and might facilitate the combination between TMV and R.

The Ca-dependent binding activity was sensitive to glycosidase (especially α-N-acetylgalactosaminidase) or lipase, suggesting that carbohydrate and lipid were important components of substance R. This lipocarbohydrate complex was precipitated in the presence of Ca++. At least, some phospholipids share this property, as shown in Table 4. It is known that binding of bivalent metal ion to phospholipid membrane results in flocculation of the membrane and that some phospholipids have a high affinity for Ca++ and Mg++. The complex formed between TMV and lipocarbohydrate was also precipitated in the presence of Ca++ and bound TMV was not released by a high concentration of NaCl. From an apparent similarity between the TMV–R–Ca++ interaction and the Ca-dependent step of TMV adsorption to membrane, it is possible that phospholipid is involved in the viral adsorption.

In the adsorption of TMV to membrane, polyornithine is supposed to modify the protein to let TMV bind easily to the above-mentioned lipocarbohydrate. As to the function of polyornithine, however, we can not exclude the possibility that TMV combines with the protein directly or through polyornithine, being followed by transfer of TMV from this protein to lipocarbohydrate. Whatever the detailed mechanism may be, it may be implied that the binding substance for TMV is lipocarbohydrate.

REFERENCES