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The antigenicity of tobacco mosaic virus

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The antigenic properties of the tobacco mosaic virus (TMV) have been studied extensively for more than 50 years. Distinct antigenic determinants called neotopes and cryptotopes have been identified at the surface of intact virions and dissociated coat protein subunits, respectively, indicating that the quaternary structure of the virus influences the antigenic properties. A correlation has been found to exist between the location of seven to ten residue-long continuous epitopes in the TMV coat protein and the degree of segmental mobility along the polypeptide chain. Immunoelectron microscopy, using antibodies specific for the bottom surface of the protein subunit, showed that these antibodies reacted with both ends of the stacked-disk aggregates of viral protein. This finding indicates that the stacked disks are bipolar and cannot be converted directly into helical viral rods as has been previously assumed. TMV epitopes have been mapped at the surface of coat protein subunits using biosensor technology. The ability of certain monoclonal antibodies to block the cotranslational disassembly of virions during the infection process was found to be linked to the precise location of their complementary epitopes and not to their binding affinity. Such blocking antibodies, which act by sterically preventing the interaction between virions and ribosomes may, when expressed in plants, be useful for controlling virus infection.

Keywords: antigenic structure; epitopes; immunoelectron microscopy; infectivity neutralization; monoclonal antibodies; stacked disks

1. INTRODUCTION

The antigenic properties of tobacco mosaic virus (TMV) have been studied extensively in many laboratories for more than 50 years (Rappaport 1965; Benjamini 1977; Van Regenmortel 1986). TMV is an excellent immunogen and antibodies to the virus can be readily obtained by immunization of experimental animals. By the 1930s, it had already been observed that antiserum raised against TMV was able to neutralize the infectivity of the virus, and in the following decade quantitative aspects of the reaction of TMV particles with specific antibodies were analysed in considerable detail by means of precipitin tests (Rappaport 1965). After the TMV coat protein (TMVCP) had been sequenced (Anderer *et al.* 1960; Tsugita *et al.* 1960), it became possible for the first time to locate the antigenic sites of a viral protein at the molecular level. Two antigenic sites of TMVCP were singled out for analysis in these initial studies: the C-terminal region corresponding to residues 153–158 (Anderer 1963) and the disordered loop region corresponding to residues 93–112 (Benjamini *et al.* 1964, 1965; Young *et al.* 1966).

2. NEUTRALIZATION OF TMV INFECTIVITY WITH ANTIPEPTIDE ANTIBODIES

Anderer and his colleagues tested peptides obtained by tryptic degradation of TMVCP for their ability to inhibit the precipitin reaction between virus and antibody, and showed that the C-terminal hexapeptide possessed the greatest activity. This hexapeptide, coupled to bovine serum albumin, was used to raise antibodies in rabbits, and the resulting antipeptide serum was found to precipitate the virus and neutralize its infectivity (Anderer 1963; Anderer & Schlumberger 1965). The specificity of the reaction was demonstrated by the fact that the neutralizing activity of the antiserum could be abolished by prior incubation of the antiserum with the hexapeptide. The neutralization assay consisted of mixing infectious virus with antiserum and assessing whether the number of local lesions obtained after inoculating tobacco leaves with the mixture was reduced compared with the number obtained with untreated virus. This local-lesion assay in tobacco is similar in principle to the plaque reduction test used in animal virology for demonstrating antibody-induced neutralization of animal viruses (Dimmock 1993; Rappaport 1965). Since natural peptide fragments as well as synthetic peptides were used in this work, Anderer and his colleagues should be credited with the discovery that synthetic peptides are able to elicit antibodies that neutralize viral infectivity. The significance of these findings for the development of synthetic vaccines was only recognized 15 years later when similar results were obtained with animal viruses (for reviews, see Arnon 1987; Arnon & Van Regenmortel 1992; Nicholson 1994).

Antibodies obtained by immunizing animals with the conjugated C-terminal tripeptide of TMVCP were also found to neutralize viral infectivity. An even more unexpected finding was the demonstration that antibodies prepared against the single terminal threonine residue of TMVCP coupled to a carrier protein were also able to precipitate the virus (Anderer & Schlumberger 1966; Anderer *et al.* 1967). This result is probably due to the particularly exposed location of the C-terminal residue in TMVCP (Bloomer *et al.* 1978; Champness *et al.* 1976). The specificity of these anti-threonine antibodies was

demonstrated by the fact that they failed to precipitate a related tobamovirus in which the C-terminal residue was serine instead of threonine (Anderer & Schlumberger 1966).

3. INTACT TMV PARTICLES AND DISSOCIATED COAT PROTEIN SUBUNITS HARBOUR DIFFERENT ANTIGENIC DETERMINANTS

Studies in the 1950s showed that the antigenic properties of intact TMV particles were different from those of dissociated coat protein subunits. Some of the antibodies present in TMV antisera were found to cross-react with dissociated coat protein, whereas other antibodies reacted only with intact virions (Jeener *et al.* 1954; Takahashi & Ishii 1952). It was suggested by Aach (1959) that the virion-specific antibodies recognized antigenic determinants that arose by juxtaposition of residues from neighbouring protein subunits. Because the state of aggregation of the viral subunits influenced their antigenic properties, a number of terms were introduced to distinguish between the different antigenic determinants found at the surface of virions and of dissociated subunits (Van Regenmortel 1966). Since antigenic determinants were referred to as epitopes (Jerne 1960), the new epitopes that arise in the polymerized viral subunits as a result of the quaternary structure were called 'neotopes'. Neotopes may be constituted by residues from neighbouring subunits that are recognized as a single entity by certain antibodies. On the other hand, since the intersubunit bonds that keep viral subunits together in the virus particle alter the conformation of the subunits, new conformational epitopes (i.e. neotopes) appear in the virions that are not present in the protein monomers. In practice, the structural basis of neotope specificity is nearly always unknown, so the label neotope is given to any epitope of the virus that is absent in the constituent monomeric subunits.

When subunits polymerize, a portion of the protein surface becomes buried and this leads to the disappearance of certain epitopes found on the monomeric viral protein. Such epitopes, which have been called cryptotopes (Jerne 1960; Van Regenmortel 1966), become accessible to antibodies only after dissociation of the virus particle.

A third label, metatope, was introduced for epitopes found in both dissociated and polymerized forms of the viral protein (Van Regenmortel 1966, 1990). Initially, the existence of antibodies specific for neotopes, cryptotopes and metatopes was established by cross-absorption experiments (Aach 1959; Takahashi & Gold 1960). After absorbing TMVCP antisera with virus particles, residual antibodies reacting with dissociated subunits and specific for cryptotopes were shown to be present. Conversely, TMV antiserum still reacted with the neotopes present on virions after being depleted by cross-absorption of all antibodies reacting with protein subunits. With the advent of hybridoma technology, it became easier to distinguish these different antigenic specificities with monoclonal antibodies (Al Moudallal *et al.* 1982; Dore *et al.* 1988) and to map the position of neotopes, metatopes and cryptotopes on the surface of viral antigens (Dore *et al.* 1990; Saunal & Van Regenmortel 1995*b*).

It should be noted that not all enzyme-linked immunosorbent assay (ELISA) formats are equally suitable for establishing whether monoclonal antibodies (Mabs) react with neotopes, metatopes or cryptotopes. When the ELISA plate is coated with a virus preparation in carbonate buffer, pH 9.6, virions tend to dissociate into subunits and these will bind preferentially to the plastic of the microtitre plates. In this case, the antigen assayed in the ELISA will be dissociated protein instead of intact virions. The nature of the antigen that reacts in ELISA can be visualized when the assay is carried out on electron microscope grids deposited at the bottom of microtitre wells, and the reacting antigen is subsequently identified by immunoelectron microscopy after replacing enzyme-labelled antibodies with gold-labelled Mabs (Dore *et al.* 1988). When Mabs are selected in a hybridoma fusion experiment using an ELISA format in which plates are coated with virus in pH 9.6 buffer, the selected antibodies will usually be specific for cryptotopes or metatopes; Mabs specific for neotopes will tend to be discarded. To select anti-neotope Mabs, it is necessary to use microtitre plates first coated with a layer of virus-specific antibodies, which will present intact virions to the Mabs (Dore *et al.* 1988).

Immunoelectron microscopy using gold-labelled antibodies has been used to visualize the location of neotopes and metatopes on TMV particles (figure 1). Most Mabs raised against TMVCP were found to be specific for metatopes (Al Moudallal *et al.* 1985) and were located at one of the two extremities of viral rods. By partly uncovering the 5′ end of the RNA in virus particles by the action of 6 M urea, it could be shown that these metatopes were located at the end of the virion containing the 5′ end of the RNA (Dore *et al.* 1990).

It was also shown by immunoelectron microscopy that these anti-metatope antibodies reacted with both ends of stacked-disk aggregates of TMVCP. This implies that the same face of the subunit, i.e. the one containing the two helices corresponding to residues 73–89 and 115–135, is exposed at both ends of the stacked disks (Dore *et al.* 1990). This finding indicates that the stacked disks are bipolar and cannot be converted directly into helical viral rods, as had been assumed for many years (Bloomer & Butler 1986; Durham *et al.* 1971; Butler, this issue). These results also explain why so many anti-metatope Mabs, recognizing the same surface of the subunit, have been obtained from the hydridoma fusion experiment with mouse spleen cells. The mice used for hybridoma production had been immunized with preparations of highly concentrated TMVCP, which contained stacked disks, and it is the presence of the same subunit surface at both faces of the disk that led to the predominance of Mabs specific for this surface (Al Moudallal *et al.* 1985).

Since these anti-metatope antibodies bind to the extremity of viral rods, which is known to become disassembled first during the infection process (Wilson 1984), experiments were done to assess whether these antibodies were able to block the disassembly of virions and the translation of viral RNA. It was found that about half of the anti-metatope antibodies that were analysed strongly inhibited disassembly and RNA translation, while the others inhibited only weakly or not at all (Saunal *et al.* 1993).

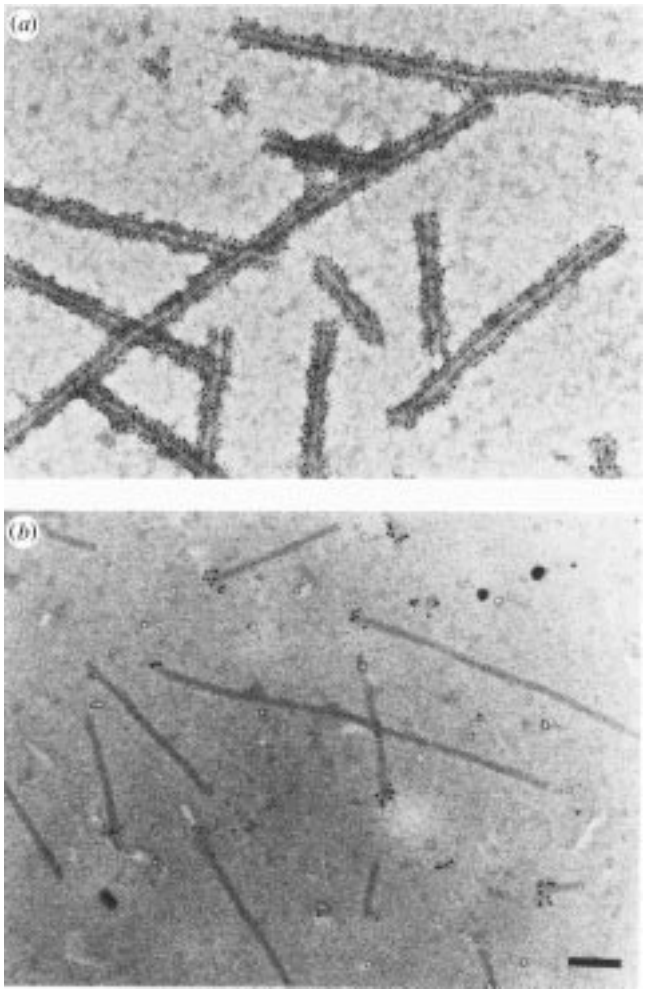

Figure 1. Electron micrographs showing the binding of Mabs to TMV adsorbed on antibody-coated grids. Bound Mabs were revealed by gold-labelled antiglobulin reagent. Note that most TMV particles are aggregated end to end. (*a*) Anti-neotope Mab 253P (1/50 000) can be seen to bind over the entire length of virus particles. (*b*) Anti-metatope Mab 16P (1/20 000) binds only to one extremity of the particles. The bar represents 150 nm (Dore *et al*. 1988).

4. IDENTIFICATION OF CONTINUOUS AND DISCONTINUOUS EPITOPES OF TMV

Epitopes are usually classified as either continuous or discontinuous, depending on whether the amino-acid residues in the epitope are contiguous in the polypeptide chain or not. The label 'continuous epitope' is given to any short linear peptide fragment of a protein that can bind to antibodies raised against the intact protein. Usually the antibodies cross-react only weakly with the peptide because the fragment does not retain the conformation present in the folded protein. In addition, the peptide often represents only a portion of a more complex epitope.

Discontinuous epitopes comprise the vast majority of epitopes found in proteins. They consist of clusters of atoms from residues that are not contiguous in the sequence but are brought near one another by the folding of the polypeptide chain. When the protein is denatured or fragmented into peptides, the residues that make up the discontinuous epitope are scattered and each individual component of the epitope is no longer recognized by the antibody. Sometimes, an antibody to a discontinuous epitope may still react with a linear peptide representing only a portion of the epitope, in which case the peptide will be called a continuous epitope although it actually corresponds to part of a discontinuous epitope (Van Regenmortel 1992).

Table 1. *Continuous epitopes identified in dissociated TMVCP*

position in sequence	detected with polyclonal antisera	with monoclonal antibodies	correlated with segmental mobility[a]	reference
1–10	+	–	+	Altschuh *et al*. 1983
19–32	+	–	+	Al Moudallal *et al*. 1985
34–39	+	–	+	Altschuh *et al*. 1983
55–61	+	–	+	Altschuh & Van Regenmortel 1982
62–68	+	–	+	Milton & Van Regenmortel 1979
80–90	+	+	+	Al Moudallal *et al*. 1985
105–112	+	+	+	Benjamini 1977
115–134	+	+	–	Al Moudallal *et al*. 1985
134–146	+	+	–	Al Moudallal *et al*. 1985
153–156	+	+	+	Anderer 1963

[a]Data from Westhof *et al.* (1984)

A total of ten continuous epitopes have been identified in TMVCP, using fragments of the coat protein obtained by enzymatic cleavage as well as synthetic peptides (table 1). The antigenic activity of the peptides was measured by radioimmunoassay (Benjamini 1977), inhibition of complement fixation (Milton & Van Regenmortel 1979) and enzyme immunoassay (Altschuh & Van Regenmortel 1982). The seven continuous epitopes of TMVCP that were seven to ten residues long were found to correspond to regions of the viral protein that were shown by X-ray crystallography to possess a high segmental mobility (Westhof *et al.* 1984). This correlation between antigenicity and mobility along the peptide chain has also been found in other proteins (Tainer *et al.* 1984, 1985; Westhof *et al.* 1984) and has been used to develop algorithms for predicting the location of continuous epitopes in proteins (Karplus & Schulz 1985; Parker *et al.* 1986; Pellequer *et al.* 1991). The correlation probably arises because flexible segments in proteins tend to correspond to N- and C-terminal segments (Thornton & Sibanda 1983) and to surface loops that are more likely to mimic the conformation of peptides in solution than regions of constrained secondary structure. Antibodies specific for elements of secondary structure such as helices also exist, but they are only detected when longer, structured peptides are used in the immunoassays (Al Moudallal *et al.* 1985; Van Regenmortel 1986).

Discontinuous epitopes in proteins are usually identified by X-ray crystallography of antigen–antibody complexes (Braden & Poljak 1995). In the case of TMV, the presence of discontinuous epitopes at the surface of virions was demonstrated by analysing the ability of Mabs prepared against intact virions to cross-react with viral mutants, with other tobamoviruses, and with dissociated viral subunits (Altschuh *et al.* 1985). Using computer-generated images of the surface residues of TMVCP, it was possible to identify clusters of residues that are probably part of discontinuous epitopes (Dore *et al.* 1987).

5. MAPPING OF TMV NEOTOPES AND METATOPES

Several cryptotopes of TMVCP have been located fairly precisely because the corresponding anti-cryptotope Mabs reacted with natural or synthetic fragments of the viral protein (Benjamini 1977; Trifilieff *et al.* 1991; Van Regenmortel 1986). This approach to epitope mapping was not feasible with neotopes and metatopes because none of the corresponding Mabs reacted with any peptide fragments of TMVCP. Some information on the location of a limited number of neotopes and metatopes on TMV particles was obtained by immunoelectron microscopy. Anti-neotope antibodies were found to react along the entire length of the viral rods, whereas some anti-metatope antibodies reacted only with one extremity of the virus particle (Dore *et al.* 1988, 1990).

The mapping of epitopes on the surface of proteins is usually done by double-sandwich ELISA, using all possible pairwise combinations of a panel of Mabs. If the second labelled Mab is able to bind to the protein antigen captured by the first Mab, it is concluded that the two Mabs bind to two distinct, non-overlapping epitopes. However, difficulties can be encountered when this approach is used with viral subunits since these tend to adsorb strongly to the plastic of microtitre plates. When TMVCP is captured on an ELISA plate by a first layer of Mabs adsorbed to the plastic, it is difficult to totally prevent additional non-specific adsorption of the viral protein to the plastic. For this reason, Mabs are usually tested as the second antibody in ELISA by measuring their capacity to react with TMVCP trapped by a first layer of polyclonal antibodies (Dekker *et al.* 1989). However, in such an ELISA format, it can never be excluded that the conformation of the viral protein is altered by the initial binding of the capturing antibody. Such a conformational change may then allow the second Mab to bind the immobilized TMVCP, although it would have been unable to react with free TMVCP. This phenomenon occurred in initial mapping experiments in which several anti-TMV Mabs (7V, 42P, 67P and 249P) were identified as anti-metatopes (Al Moudallal *et al.* 1985; Altschuh *et al.* 1985). Later work using biosensor technology showed that these antibodies were actually anti-neotopes and were unable to react with free TMVCP molecules (Saunal & Van Regenmortel 1995*b*). Such erroneous interpretations arise because it is not possible, using ELISA, to visualize each of the successive binding steps in the assay, as is done for instance in a biosensor instrument. In double-antibody-sandwich ELISA, the binding of the second antibody is needed to reveal the initial binding of the first capturing antibody, and it cannot be excluded that a conformational change occurred in the antigen as a result of the reaction with the first antibody. Using the biosensor technology, it was indeed possible to show that TMVCP captured by a first anti-metatope Mab underwent a conformational change, which then allowed it to be recognized by an anti-neotope Mab (Dubs *et al.* 1992; Saunal & Van Regenmortel 1995*b*).

The introduction in 1990 of biosensors based on surface plasmon resonance has greatly simplified the mapping of epitopes (Daiss & Scalice 1994; Fägerstam *et al.* 1990). Biosensor instruments make it possible to visualize the antigen–antibody binding process as a function of time by following the increase in refractive index that occurs when one of the interacting partners binds to its ligand immobilized on the surface of a sensor chip. The concentration of molecules that bind to the sensor-chip surface (expressed as resonance units or RU) is monitored continuously over time and is registered as a sensorgram. None of the reactants needs to be labelled, which avoids possible artefactual changes in binding properties resulting from labelling (Fägerstam & Karlsson 1994; Malmqvist 1993). The most widely used biosensor instrument is the BIAcore™ (BIAcore AB, Uppsala, Sweden).

Since the binding stoichiometry observed in the BIAcore is easily calculated from the molar ratio of bound antibody/antigen, it is a simple matter to ascertain whether Mabs bind to only one extremity of TMV particles or along the entire length of virions. Such an experiment is illustrated in figure 2, which shows the binding of anti-metatope Mabs 5V and 25P to TMV particles first immobilized by an anti-neotope Mab. The molar ratio of bound Mab 25P/TMV was found to be about ten, indicating that the antibody bound only to one end of the virion. The molar ratio of Mab 5V/TMV was about 300, indicating that this antibody bound to the entire surface of the particle. Although the anti-metatope Mabs studied earlier by immunoelectron microscopy (Dore *et al.* 1988, 1990;) were all found to react with only one surface of the subunit (surface E in figure 3), biosensor experiments revealed that other anti-metatope antibodies recognized surface A of TMVCP (table 2 and figure 3).

When anti-neotope Mabs, characterized by their inability to capture dissociated viral subunits in the BIAcore, were analysed in the same way, some of the antibodies gave high molar ratios of bound Mab/TMV indicating that they were binding to surface A. Others gave ratios of only 5–15 even when a considerable excess of Mab was used, indicating that the antibodies were binding to surface E (Saunal & Van Regenmortel 1995*a*).

A summary of data obtained with 20 Mabs showing the location of their binding sites on TMV is presented in table 2. In such assays, two epitopes will be recognized as different only if they are far enough apart to allow simultaneous binding of the two Mabs. In some cases, however, Mabs directed against distinct but neighbouring epitopes will be prevented from binding concurrently to the antigen surface because of steric hindrance.

The presence of previously undetected metatopes on surface A of the viral subunit increased the range of two-site binding assays that could be performed with TMVCP monomers. This is due to the fact that it became possible to

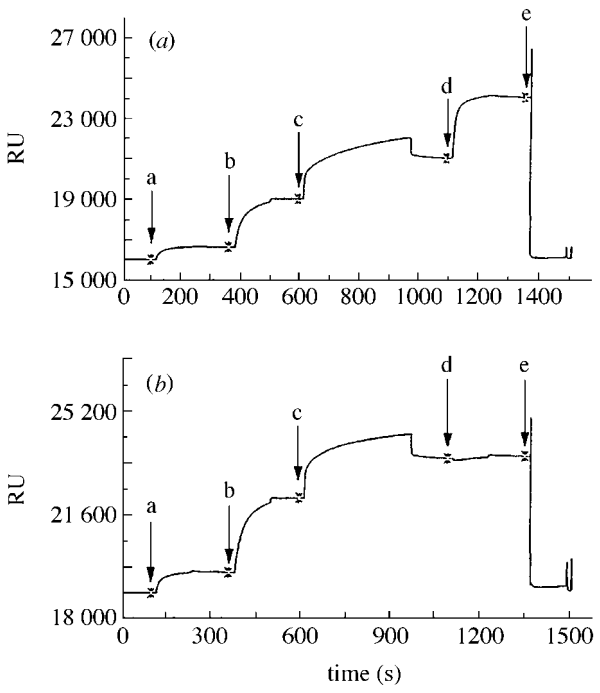

Figure 2. Sensorgrams used for determination of binding sites of anti-neotope and anti-metatope antibodies on TMV particles. Phase (a) corresponds to the injection of capture anti-neotope Mab 253P (ascitic fluid diluted 1/10). About 700 RU were bound. Phase (b) corresponds to 10 µl of TMV (100 µg ml^{-1}) injection (*ca.* 2500 RU). Phase (c) corresponds to the saturation of the rabbit anti-mouse globulins by means of one 30 µl injection of non-specific ascitic fluid (*ca.* 1500–2500 RU). Phase (d) corresponds to the binding of Mabs 5V and 25V (10 µl injection of 1 µM Mab) along the entire surface of the virus particle (sensorgram (*a*) with a stoichiometry of Mab 5V/TMV of 300) or only at one extremity of the rod (sensorgram (*b*) with a stoichiometry of Mab 25V/TMV of 10), respectively. Phase (e) corresponds to the regeneration phase with 100 mM HCl (10 µl). A flow rate of 5 µg min^{-1} was used during phases (a) to (e).

present TMVCP in such assays in two different orientations using capturing anti-metatope Mabs specific for surfaces A and E, respectively (Saunal & Van Regenmortel 1995*a*). Whenever pairs of anti-metatope Mabs binding, respectively, to surface A or E were used in the same assay, they were found to bind concurrently to the same TMVCP molecule. In addition, some pairs of Mabs recognizing the larger surface E were able to bind simultaneously to the same viral subunit, but no case was observed where two Mabs of type A bound to the same TMVCP molecule (figure 4).

In some cases, TMVCP presented by an anti-metatope Mab became capable of binding to an anti-neotope Mab specific for a different surface of the subunit, presumably because a neotope conformation had been induced in the monomeric viral subunit. For instance, anti-neotope Mabs 7V and 249P of type A could bind to TMVCP presented by an anti-metatope Mab of type E (16P or 151P) and in addition some anti-neotope Mabs of type E (42P and 67P) were able to bind to TMVCP presented by some anti-metatope Mabs of type A (5V or 19V) (Saunal & Van Regenmortel 1995*b*). The induction of a neotope conformation in monomeric TMVCP subunits following binding of the subunit to a first antibody is likely to mimic at least partly the conformational change that occurs in the subunits when they assemble into a quaternary structure. It should be noted, however, that certain neotope specificities cannot be induced in the monomer in this manner as shown, for instance, by the inability of anti-neotope Mabs 4P and 18V to recognize TMVCP presented by any of the anti-metatope Mabs. The most likely explanation is that such anti-neotope antibodies recognize an epitope formed by the juxtaposition of residues from two neighbouring subunits.

The refined mapping of TMV epitopes achieved with the biosensor technology made it possible to investigate if there was any correlation between the location of epitopes on TMVCP and the capacity of the corresponding antibodies to inhibit co-translational disassembly (Wilson 1984). Since the binding affinity and kinetics of antibodies are easily measured in the BIAcore (Karlsson & Roos 1997; Saunal *et al.* 1997), it was also possible to establish if there was any correlation between the affinity and inhibitory capacity of the antibodies. Experiments with anti-metatope Mabs showing the greatest variation in inhibition capacity led to the conclusion that there was no correlation between the capacity of antibodies to inhibit co-translational disassembly of TMV and their binding affinity (Saunal & Van Regenmortel 1995*b*).

During the course of two-site binding assays, continuous dissociation and reassociation of the binding partners occurs. When the kinetic dissociation rate constant (k_d) of the first capturing Mabl is about 10^{-3} s^{-1}, approximately half of the trapped antigen will become dissociated during the 10 min of Mab2 injection. On the dissociated antigen molecule, the region of the surface previously occupied by Mabl may then bind to Mab2 and this will prevent reassociation of the antigen to Mabl, resulting in a lower RU response. In view of this dynamic competition between different Mabs, this type of epitope mapping has been called kinetic mapping (Saunal & Van Regenmortel 1995*b*). If the k_d value of Mabl is faster than 10^{-2} s^{-1}, no meaningful data can be obtained in such experiments, since practically all antigen molecules will dissociate during the time frame of the experiment.

The results of two-site binding assays were used to establish the relative position of epitopes on surfaces E and A of the TMVCP subunit (figure 4). The combining sites of the antibodies were assumed to cover a minimum surface of 600 Å in a circular footprint (Braden & Poljak 1995). Usually pairwise interaction data give information only on the relative positions of epitopes (Daiss & Scalice 1994). However, in the case of TMVCP, it was possible to construct a map depicting the actual physical location of the epitopes on the subunit surface. Use was made of the known specificity for surface A or E of each Mab, and of the fact that Mab 29V specific for surface A interfered with the binding of Mabs 16P and 47P specific for surface E.

The epitope map shown in figure 4 was useful for understanding the mechanism by which certain anti-TMV antibodies are able to block the disassembly of TMV by ribosomes (Saunal *et al.* 1993). Mabs 25P 151P 167P 181P and 188P which possessed the strongest inhibitory capacity were found to bind to the region of surface E that is closest to the central axis of polymerized

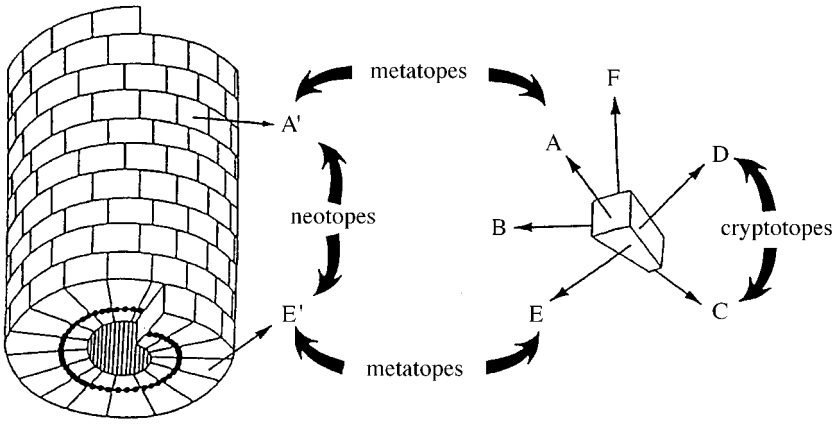

Figure 3. Schematic model of the protein subunits of TMV in monomeric form and in the virus particle. Neotopes are found on surface A′ and E′, metatopes are found on surfaces A, A′, E and E′, and cryptotopes are found on surfaces B, C and D. The type of epitope present on surface F has not yet been defined. The E′ extremity of the virion contains the 5′ end of the RNA (Saunal & Van Regenmortel 1995*a*).

Table 2. *Location of metatopes and neotopes recognized by various Mabs on different surfaces of TMV and TMVCP* (*see figure 3*)[a]

	surfaces A and A′ molar ratio bound Mab/TMV *ca.* 300	surfaces E and E′ molar ratio bound Mab/TMV *ca.* 10
Mabs able to capture TMVCP	anti-metatope Mabs of type A: 5V, 6V, 17V, 19V, 29V, 236P	anti-metatope Mabs of type E: 16P, 25P, 47P, 98P, 151P, 173P, 181P
Mabs unable to capture TMVCP	anti-neotope Mabs of type A: 7V, 18V, 249P, 253P	anti-neotope Mabs of type E: 4P, 42P, 67P

[a] The four categories of Mabs are defined by their ability to capture TMVCP subunits in the BIAcore and by their stoichiometry of binding to intact TMV particles.

TMVCP. Since this part of the subunit surface is known to interact with the viral RNA, it seems likely that the inhibitory Mabs act by sterically preventing the interaction between RNA and ribosomes (Saunal & Van Regenmortel 1995*b*). Such antibodies, when expressed in plants, may be useful for controlling virus infection. The mode of action of the inhibitory antibodies resembles the neutralization mechanism operating when antibodies to animal viruses prevent virions from interacting with cellular receptors (Dimmock 1993).

6. DETERMINATION OF THE AFFINITY OF ANTIBODIES TO TMV

Knowledge of the affinity of antiviral antibodies is important for the proper design of immunoassays and for understanding the biological activity of antibodies, for instance in the neutralization of virus infectivity (Dimmock 1993; Van Cott *et al.* 1994). In the past, virus–antibody binding data were interpreted in terms of monovalent binding of antibody, mainly because the kinetics for bivalent binding to the same virus particle were thought to be unfavourable (Fazekas de St Groth 1962). However, it seems that in some cases the presence of large numbers of identical viral subunits at the surface of virions may actually favour bivalent antibody binding. The uncertainty concerning the monovalent or bivalent nature of antibody binding to virus particles was resolved in the case of TMV when it was shown that both types of binding occurred depending on the antigen–antibody ratio (Van Regenmortel & Hardie 1976).

Initial studies of TMV–antibody interactions by means of classical Scatchard plots had used as antigenic valency the number of identical protein subunits (2130) in the virion. This gave rise to linear plots, which were interpreted erroneously as evidence for homogeneous antibody binding (Mamet-Bratley 1966; Urbain *et al.* 1972). However, when Scatchard plots of the type f/d versus nf were used in the analysis instead of classical r/c versus sr plots (Day 1990), the antigenic valency of the virus could be determined experimentally and was found to be 800 instead of 2130, the difference being due to steric hindrance (Hardie & Van Regenmortel 1975). The symbols used in these two types of Scatchard plots are the following: f, ratio of bound antibody to total antigen; d, free antibody concentration; r, ratio of bound antigen to total antibody; c, free antigen concentration; n, antibody valence; s, antigen valence. As expected with polyclonal antibodies, heterogeneous antibody binding was observed in the f/d versus nf plots and evidence for both monovalent and bivalent antibody binding could be obtained (Van Regenmortel & Hardie 1976) (figure 5).

At low antibody–antigen ratios, the two combining sites of an IgG molecule bind to identical epitopes on the same virus particle, a situation known as monogamous bivalent binding characterized by a high functional avidity. These results were obtained from binding assays in which free antibody was separated from virus–antibody complexes by centrifugation and quantitated by spectrophotometry. More recently, it was shown that the same type of Scatchard analysis could be done at much lower antibody concentration by measuring the amount of free antibody at equilibrium by ELISA titration (Azimzadeh & Van Regenmortel 1991; Azimzadeh *et al.* 1992). It is important to assess if antibodies bind in a monovalent or bivalent manner since the calculated antibody affinity

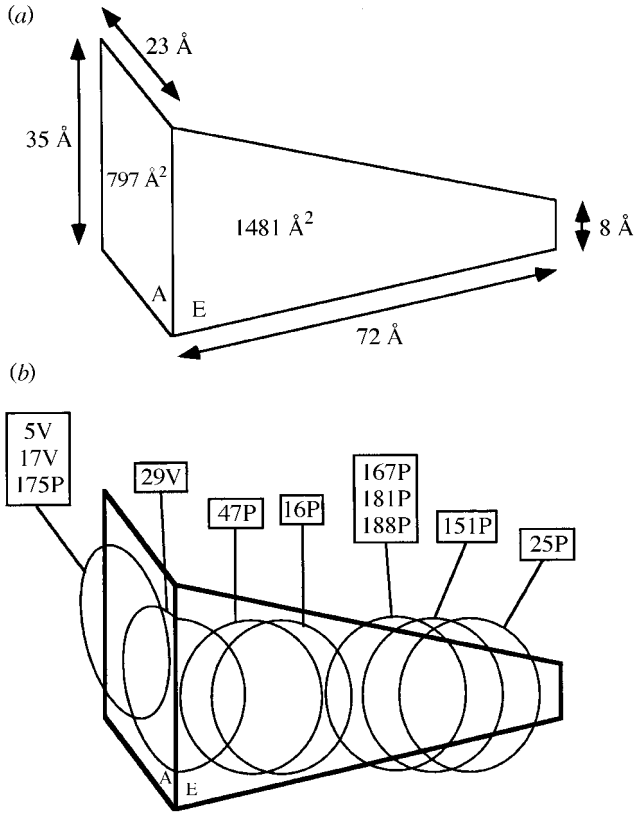

Figure 4. (*a*) Schematic model of surfaces A and E of the TMVCP subunit. (*b*) Schematic epitope map constructed from pairwise interaction data with Mabs. Each antibody-combining site was assumed to cover 600 Å^2 in a circular footprint. Mabs 25P, 151P and 181P inhibit strongly the cotranslational disassembly of the virus. Mabs 188P, 167P, 17V and 29V possess less inhibitory activity, while the other Mabs do not inhibit at all (Saunal & Van Regenmortel 1995*b*).

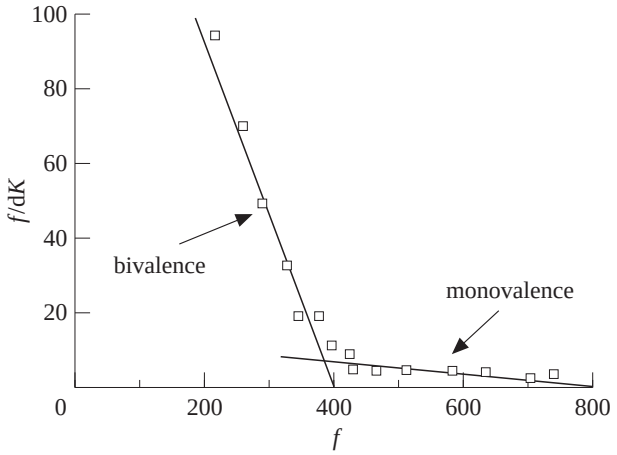

Figure 5. Binding data of the interaction between TMV and Mab 107P (intact IgG molecules). Monovalent binding of antibody occurs preferentially in the region $f=400$ to $f=800$ and bivalent binding in the region $f=0$ to $f=400$. The two portions of the plot extrapolate to $s=773$ and $s/2=408$, respectively. Data were obtained as described by Azimzadeh & Van Regenmortel (1991). From Pellequer & Van Regenmortel (1993*a*).

may be about two orders of magnitude lower in the case of monovalent binding (Van Regenmortel 1997).

7. THE ANTIGENIC VALENCY OF TMV

Antigenic valency is defined as the maximum number of epitopes per antigen that can be simultaneously occupied by antibody molecules (Singer 1965). According to this definition the valency is a constant parameter and not a variable influenced by the ratio of antibody to antigen (Van Regenmortel 1988). Although some authors have assumed that the antigenic valency of TMV is equal to the number of viral subunits, i.e. 2130 (Anderer *et al.* 1971; Mamet-Bratley 1966), the correct value is 800 (Azimzadeh & Van Regenmortel 1991). The difference between these two values is due to the fact that each antibody-combining site covers the surface of approximately three viral subunits. The ratio $2130/800=2.7$ is close to the ratio of the molecular weights of a Fab fragment and the TMV subunit ($50\,000/17\,500=2.9$). It should be emphasized that the antigenic valency of the TMV particle is 800 even under conditions of extreme antigen excess when only a few antibody molecules are bound to each particle and each antibody has the choice to bind to any of the 2130 free subunits.

Cowan & Underwood (1988) have developed a mathematical model that describes the binding of antibody to a multivalent antigen under conditions of steric hindrance. This model predicts that the steric-hindrance effect necessarily results in curved Scatchard plots, also in the case of homogeneous binding of Mabs. When Mabs bind to TMV particles, the model predicts that f/d versus f plots will approximate to a quadratic curve of the type shown in figure 6. The intercept of the curve with the x-axis gives the antigenic valency s and its intercept with the y-axis gives the value uK where u is the total number of epitopes ($u=2130$) on the TMV particle that could be recognized by the Mab. In figure 6, the straight lines correspond to situations that would prevail in the absence of steric hindrance. The line extrapolating to $f=2130$ on the x-axis represents the hypothetical situation where an antibody would have the same size as the viral subunit (17 500, resulting in no steric hindrance. The line extrapolating to $f=800$ corresponds to the situation where the viral subunit would have a size similar to that of a Fab fragment (50 000), which again would remove the steric effect. According to the analysis of Cowan & Underwood (1988), the usual procedure for calculating affinity constants by fitting straight lines to experimental f/d versus f data gives rise to erroneous affinity values, the error being larger, the greater the ratio u/s.

When antibody–TMV binding data were analysed in terms of the Cowan & Underwood model (1988), it was found that the experimental data fitted a quadratic curve better than a straight line (Pellequer & Van Regenmortel 1993*a*). However, the error in affinity-constant determination caused by fitting a straight line instead of a curve leads to values that are wrong by only a factor of two, a difference that lies within the error of current methods of antibody affinity measurements. In all TMV data sets that were analysed (Pellequer & Van Regenmortel 1993*a*) the value of u/s calculated by extrapolation of the fitted curves to the y-axis was close to the value 2.7 predicted by the Cowan & Underwood (1988) model, and better fits were always obtained when experimental points were

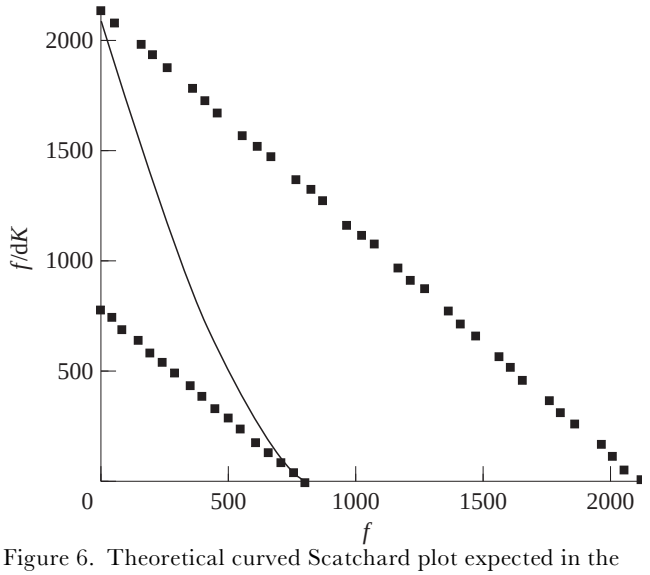

Figure 6. Theoretical curved Scatchard plot expected in the TMV–antibody system from the Cowan & Underwood (1988) model. The virus particle is known to possess 2130 identical subunits and the antigenic valency has been determined experimentally as $s=800$. The two dashed lines correspond to hypothetical cases where steric hindrance would be absent when the antibody Fab fragment is as small (17 500 Da) as the viral subunit ($f/dK=2130$) or when the viral subunit is as large (50 000 Da) as the Fab fragment ($f/dK=800$) (Pellequer & Van Regenmortel 1993*a*).

represented as curves instead of straight lines. In other virus–antibody systems, the magnitude of the steric effect and the error in affinity-constant determination is likely to be even smaller than with TMV, since the protein subunits of most viruses tend to be larger than 17 500 Da and the u/s ratio will thus be smaller. Detailed experimental procedures for measuring the affinity constant of TMV antibodies have been described in several reviews (Azimzadeh *et al.* 1992; Pellequer & Van Regenmortel 1993*a*,*b*; Saunal *et al.* 1997; Van Regenmortel 1997).

8. FINE SPECIFICITY STUDIES OF TMV EPITOPES AND PARATOPES BY RESIDUE SUBSTITUTIONS AND MUTAGENESIS

It is well known that each residue of a continuous epitope is not necessarily a residue in contact with the combining site of the antibody, in which case it will not contribute to the binding energy of interaction (Getzoff *et al.* 1988; Van Regenmortel 1994). Participation of individual residues of the epitope to the interaction can be assessed by measuring the effect of single amino-acid substitutions on the kinetic rate constants of the interaction. When epitopes of TMVCP located in the peptides 125–136 and 134–146 were analysed in this way, it was found that only a few of the residues in these peptides contributed to the energy of interaction (Altschuh *et al.* 1992; Chatellier *et al.* 1996; Zeder-Lutz *et al.* 1993). In most cases, substitutions in the peptide only affected the dissociation rate constant and not the association rate constant. Kinetic analysis using the BIAcore was also used to demonstrate the cooperative effects of multiple mutations in anti-TMV Fabs (Rauffer-Bruyerère *et al.* 1997). The results showed that certain residues located away from the binding site were able to influence the dissociation kinetics of antigen–antibody interactions. Deviations from a simple additivity effect of multiple mutations were observed with respect to dissociation kinetics and could be quantified unambiguously because of the precision of biosensor measurements, which allowed 20% differences in the dissociation rate constant to be measured in a reliable manner (Rauffer-Bruyerère *et al.* 1997).

9. USE OF ANTIGENIC PROPERTIES IN THE CLASSIFICATION OF TOBAMOVIRUSES

TMV is the type species of the genus *Tobamovirus*, which comprises a total of 13 species (Murphy *et al.* 1995). Many of these viruses were initally considered to be strains of TMV on the basis of antigenic cross-reactions. In order to quantify the antigenic similarity between different tobamoviruses, a parameter known as the serological differentiation index (SDI) was introduced (Van Regenmortel & Von Wechmar 1970). SDI values correspond to the average number of twofold dilution steps separating homologous from heterologous antiserum titres. A close correlation was found to exist between the antigenic distance between individual tobamoviruses expressed as SDI values and the degree of sequence difference in the respective coat proteins (Van Regenmortel 1975). SDI values can be obtained from precipitin tests or from ELISA experiments, which require much smaller quantities of reagents (Dubs & Van Regenmortel 1990; Jaegle & Van Regenmortel 1985). It is customary to consider that two viruses that differ antigenically by an SDI value larger than four or five correspond to separate viral species (Van Regenmortel *et al.* 1997).

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