THE CAUSE OF IRREVERSIBLE POLYMERISATION OF TOBACCO MOSAIC VIRUS PROTEIN

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1. Introduction

Protein from tobacco mosaic virus (TMV) can polymerise into many different organised structures. Some of these polymers form reproducibly and reversibly in conditions which have been described recently [1–5], but others, of which the best known is the stacked disk rod, form irreproducibly in conditions which have been only partially characterised [4, 6]. The present paper shows that the stacked disk rod, and several other polymers, result from proteolytic cleavage of the TMV polypeptide chain, thus explaining many strange observations upon the protein and providing an unusually detailed illustration of a spoilage mechanism by which a protein can lose its biological activity.

Stacked disk rods of TMV protein have been observed in many laboratories and their structure has been characterised by Finch and Klug [7]. It was originally thought that they differed only slightly from the virus-like single helix, since both types of rod have very similar intersubunit bonds and overall dimensions (see fig. 1), but it is now clear that there is a fundamental difference, since stacked disk rods are much more difficult to dissociate than single helices or any other reproducible polymers. Carpenter [6] observed that stacked disk rods formed when TMV protein was kept for weeks in alkaline solution, and that they were not in reversible equilibrium with their precursors, but required extremes of pH for dissociation.

Independently [8], I had also observed that stacked disk rods generally were the ultimate aggregate formed when alkaline TMV protein solutions were left to stand for a long time. Observation of a number of other irreproducible polymerisation states of TMV protein – "fish" [1], a single peak sedimenting at about 8 S [2], irreversibly associated short stacks of disks [3], abnormally high molecular weights in sedimentation equilibrium experiments [3], cloudiness appearing in solutions stored at alkaline pH [3], open helical rods [4], rods with an unusually wide central hole [4], and double helical rods [7] – led to the working hypothesis that some slow change (such as deamidation, cross-linking, loss of the C-terminal residue [9], or a slow change to another conformation) could occur to TMV protein in any state of reversible association, causing it to become "locked in that structure and unable to dissociate easily. Thus, fish would be locked two-layer aggregates (A-protein), stacked disk rods would be a locked and longer version of reversible stacks of disks, the single 8 S sedimentation peak would be due to a locked form of the 8 S structure normally found in reversible equilibrium with A-protein, and so on. Other authors have also observed behaviour which is inconsistent with the normal reversible polymerisation behaviour. For example, McCarthy [10] separated by gel electrophoresis several TMV protein polymers which could not have been in a state of rapid association equilibrium, and Kleczkowski [11] observed rods, whose electron micrographs look suggestive of stacked disk rods, when he irradiated TMV protein with ultraviolet light, at a pH where single helix is not stable.

Stacked disk rods have been useful experimentally, being regular polymers in which the structural details

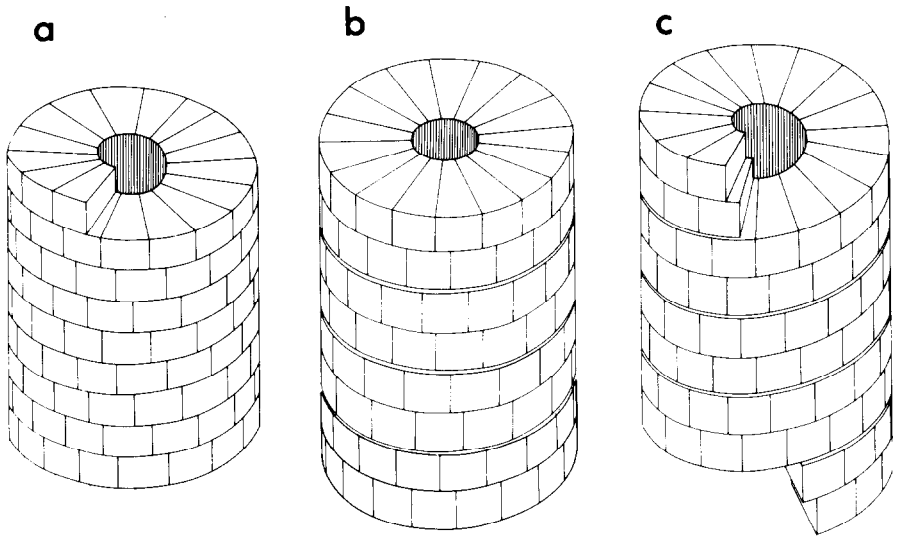

Fig. 1. Diagrammatic representation of the structure of the three best characterised types of TMV protein rods [7]. All three types of rod have a diameter of about 180 Å and a vertical distance of about 25 Å between successive layers of subunits. Intersubunit bonds are substantially the same in all these rods, differing only by small quasi-equivalent deformations. (a) Single helix. This is the reversibly polymerised rod form, stable only at mildly acid pH. Its structure is essentially the same as the protein part of the parent virus, with 16 1/3 subunits per helical turn. (b) Stacked disk. Rings of 17 subunits are associated in pairs (disks) along the length of the particle. Although the diagram shows only four disks, the rods examined in this paper are typically much longer, containing tens or hundreds of disks. The term "stacked disk rod" is applied here only to these irreversibly polymerised long rods, which must be distinguished carefully from the disks and short stacks of disks, which can be formed in well defined solution conditions. (c) Double helix. This is a two-start helix with subunits paired between the turns as in stacked disk rods.

of pairing can be studied [1, 8] and as undissociating archetypes for the titration behaviour of the two-layer aggregates [2]. On the other hand their occurrence can be a nuisance experimentally, since the circumstances of their formation would suggest "denaturation" if it were not for the regular structures observable in the electron microscope. So the present investigation sets out to answer two questions. How does the protein in stacked disk rods differ from that in normal reversible polymers? And, is there any way to form stacked disk rods controllably from normal protein?

2. Experimental

2.1. *Nature of the protein in stacked disk rods*

Some "natural" stacked disk rods for detailed study were obtained as follows. TMV protein residues from various experiments were collected and pooled over a period of months, and then dialysed against Tris buffer, pH 8.5, ionic strength 0.1, at 5°, and centrifuged at 100 000 *g* for 1 hr. The pellet was redissolved in Tris buffer and passed through a Millipore filter of nominal pore size 480 nm. In this way all those TMV protein polymers, in the approximate size range from hundreds to thousands of subunits, that did not dissociate at pH 8.5, 5°, were obtained without regard to the mechanism that had caused their stability. Examination in the electron microscope showed rods of typical stacked disk appearance, with clear striations of 50 Å periodicity, but no significant quantities of any other regular polymers or disordered protein.

Samples of these stacked disk rods were analysed by acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS). Contrary to the natural expectation that there might be covalent

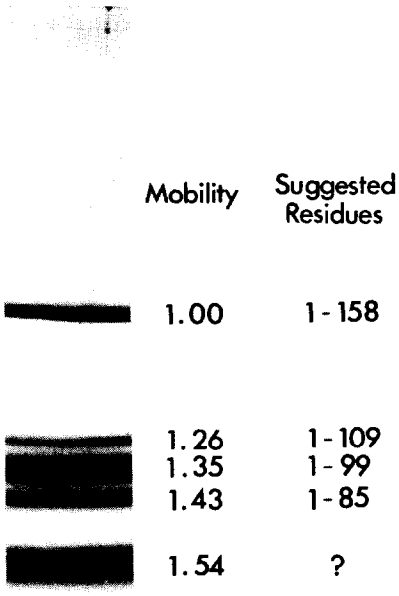

Plate 1. An SDS gel electrophoresis pattern of a natural preparation of stacked disk rods. Migration is from top to bottom and the mobilities of the bands are marked relative to that of the protein monomer, together with the deduced portions of the polypeptide chain causing them.

This photograph shows part of a large scale gel on a flat plate, run according to the system of J.V. Maizel and U.K. Laemmli (see [12]), with a 15% acrylamide separating gel in a Tris/HCl buffer, and a Tris/glycine electrode buffer. Protein bands stained with Coomassie Brilliant Blue were cut out for amino acid analysis [13] and molecular weights were estimated in the usual way [12].

Plate 2. The SDS gel electrophoresis pattern of a different preparation of stacked disk rods from that of Plate 1. Experimental conditions were as described for Plate 1.

bonds holding subunits together in the rods, the SDS gel pattern (Plate 1) shows no material larger than the TMV protein monomer (of molecular weight 17 500 daltons), but there are a few bands of material with lower molecular weights. Other samples of TMV protein containing anomalously heavy aggregates showed qualitatively similar patterns: always bands of smaller material, never any polypeptides larger than the protein monomer. For example, Plate 2 shows the pattern obtained with a sample of stacked disk rods made [7] by leaving TMV protein for three months at pH 8, ionic strength 0.6, 5°.

Control samples of fresh TMV protein always showed a single band upon SDS gels, so the low molecular weight bands must be the result of cleavage of the TMV polypeptide chain. Attempts were therefore made to locate the cleavage points in the protein sequence. The molecular weights of the fragments were estimated from their mobilities upon the gels compared with the mobilities of proteins of known molecular weights. Then the gel electrophoresis was scaled up to handle several milligrams of protein, and stained bands were cut out for amino acid analysis, as described by Butler [14]. Combining the molecular weight estimates with the amino acid analyses, and assuming that the major bands result from just one cleavage, suggests that three of the fragments indicated in Plate 1 can be identified as the results of cleavages between these residues: glycin

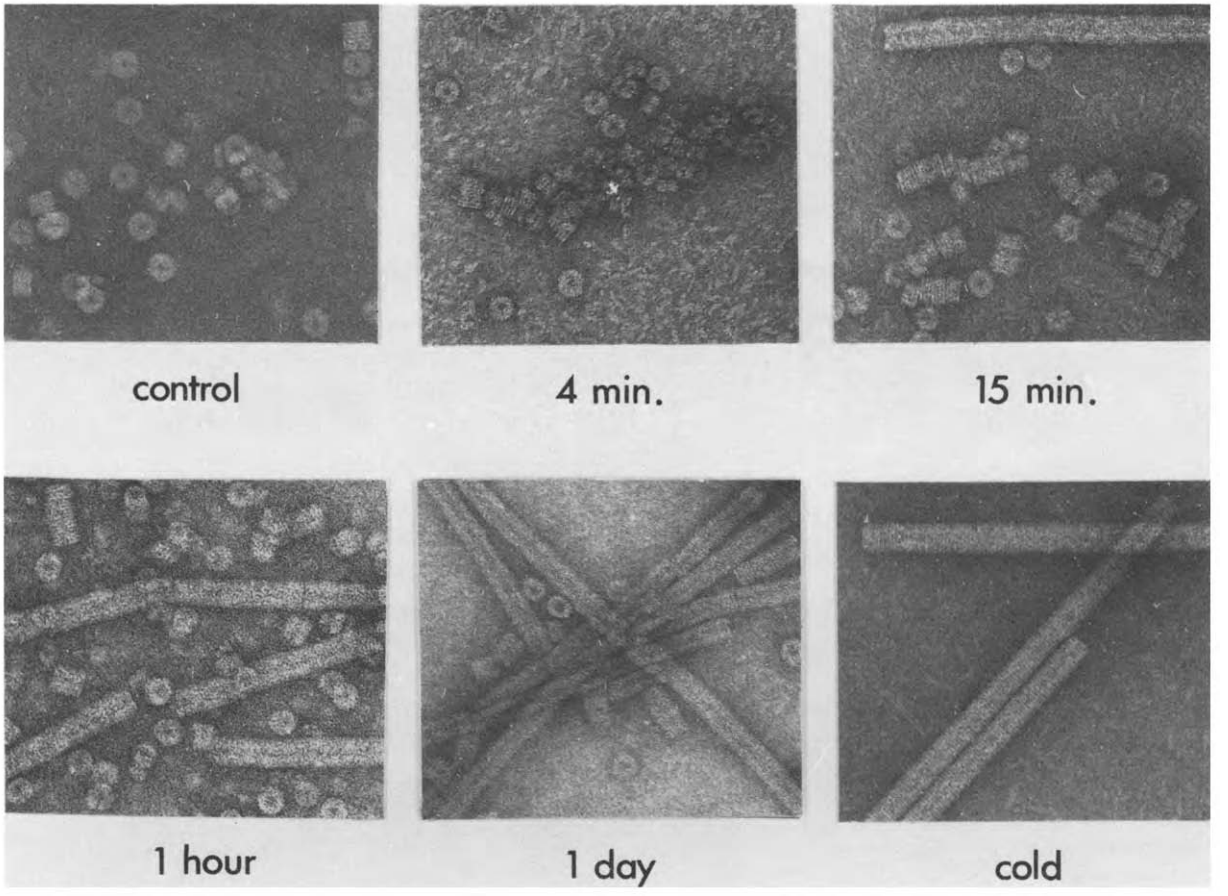

Plate 3. A series of electron micrographs showing the time course for formation of stacked disk rods by controlled proteolysis. The protein began as disks and stacks of disks, and then as proteolysis proceeded, first "fish" [1] formed, and then the number and length of rods increased steadily. After long incubation times the solution contained a mixture of very long helical rods and intermediate length stacked disk rods. Cooling the solution converted all the rods to the stacked disk form. The micrographs were kindly taken by Dr. J.T. Finch, with whom I am collaborating in a study of the formation of large irreversible polymers of TMV protein. Experimental details were as follows. A solution of TMV protein, at a concentration of 5 g/l, was dialysed for 2 days at 5° against 0.9 M NaCl 0.15 M Tris, 0.10 M HCl solution, giving mostly two-disk-stacks [3] (control). Then, trypsin was added (1:40 w/w) and the solution incubated at room temp., with samples taken for electron microscopy [4], negatively stained with uranyl acetate, at the times indicated. For the sixth micrograph a sample of the solution was cooled to 5° after 3 hr of incubation and then examined next day. The appearance of cloudiness in the solution parallelled the appearance of long rods in the micrographs. All micrographs are reproduced approx. × 180 000.

85 and alanine 86; glutamine 99 and alanine 100; alanine 110 and threonine 111. Experiments are under way to make these tentative identifications more certain, and full details will be published shortly.

2.2. *Formation of stacked disk rods by controlled protease treatment*

Besides the demonstration that stacked disk rods contain cleaved protein, it also needs to be shown that enzymatic cleavage of TMV protein can lead to the formation of large polymers. To this end, solutions of TMV protein in several states of aggregation were prepared and treated with various proteolytic enzymes. In general it was found that proteolytic cleavage did cause the formation of regular polymers, and that the initial reversible state of aggregation of the protein was more important for de-

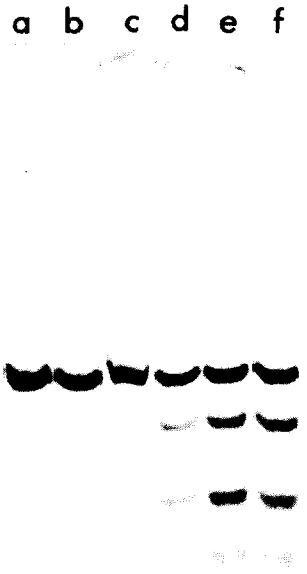

Plate 4. A series of SDS gel electrophoresis patterns showing the time course of appearance of low molecular weight bands during trypsin proteolysis. The same experiment yielded the electron micrographs in Plate 3. Samples were taken after digestion for these times: (a) zero time (control), (b) 1 min, (c) 4 min, (d) 15 min, (e) 1 hr, (f) 4 hr, and were dissociated, and also further digestion stopped, by heating to 100° for 1 min in 2% SDS, 5% mercaptoethanol. Still longer times of digestion showed no significant change from the 4 hr pattern. The control sample shows several minor bands, which remained unchanged during the tryptic digestion and presumably arose from some natural cleavage during the initial 2 days dialysis to form disks. No simple cleavage scheme can explain the appearance of three discrete bands besides monomer but the problem is under investigation.

termining its final structure than the particular enzyme used. On protease treatment, A-protein yielded mostly fish, disks yielded a mixture of fish and various types of rods, and two-disk-stacks yielded mostly long stacked disk rods. Stacked disk rod formation was caused very efficiently by some enzymes, notably trypsin and subtilisin, but not, for instance, by chymotrypsin. Pronase caused the formation of rods which had helical, not cylindrical, symmetry, but it has not been possible to tell whether they were single or double helices.

An example of the kind of behaviour that can be observed when a proteolytic enzyme controllably digests TMV protein, initially in a known state of reversible aggregation, is shown in Plates 3 and 4. In this experiment, some TMV protein, composed mostly of 2-disk-stacks, was treated with trypsin, and series of electron micrographs and SDS gel patterns show the parallel formation of long rods and protein fragments with time. Experimental details are described in the legends to the plates. This experiment clearly raises a number of interesting questions about the nature of the polymerisation process, but at present the important fact is simply that deliberate cleavage of TMV protein specifically causes long rods to form.

3. Discussion

These experiments demonstrate both that the protein in "natural" stacked disk rods has cleavages in its polypeptide chain and also that limited experiment al cleavage of the protein leads to the formation of stacked disk rods and other forms. They raise, but do not answer, the question of what is the minimum damage to the protein essential to cause "irreversible" polymerisation. For example, do the stacked disk rods made by some TMV mutants and other rod-shaped viruses, also contain breaks in the chain? The proportions of the different bands in Plate 1 suggest that stacked disk rods contain normal polypeptide chains as well as cleaved ones, and also that the C-terminal portions of the chain complementary to the provisionally identified N-terminal fragments have either been cleaved further or lost from the structure altogether. Also the rods may be more heterogeneous than their apparent uniformity in electron micrographs suggests.

The universal occurrence of stacked disk rods, and kindred polymers, in laboratories studying TMV protein suggests that proteolytic cleavage is not simply the result of bad technique or impure samples. I suggest that many of the strange results which bedevil the extensive literature about TMV protein may have been caused by undetected damage to the protein. Therefore, care must be taken with any associating system to distinguish between reproducible results obtained with native protein and chance observations obtained with damaged protein. Loss of

biological activity of many other proteins is also often thought to be due to proteolysis. The origin of the enzymes attacking TMV protein is sometimes obvious (bacterial growth is a very effective generator of unusual polymers), but more often it must be ascribed to some small contamination carried over from the plant sap absorbed to the virus rods. It is possible to protect TMV protein from attack by storing it as single helix, but it might also be possible to inhibit the enzymes, for example with diisopropyl fluorophosphate.

Limited availability of sites for proteolysis is a familiar observation among native proteins such as myosin, DNA polymerase and immunologlobulins, but TMV protein shows the additional influence of quaternary structure. Stacked disk rods, like intact virus, seem to protect their protein from further tryptic cleavage, presumably because their structure does not expose any sensitive peptide bonds to attack. Potential cleavage sites are, however, accessible on disks and A-protein.

The behaviour of TMV protein is paradoxical in that it associates more strongly into a large overall aggregate when its ultimate units become smaller. Analysis of electron micrographs shows [7] some small differences in structure between the subunits in helical and stacked disk rods. Therefore, it seems that when the integrity of the polypeptide backbone is broken, the two parts of the subunit relax into a thermodynamically more favoured conformation. The conformation of the intact chain would then permit only a weak interaction between subunits but the conformation of the cleaved chain would permit much stronger intersubunit bonds. This suggests a tenseness in the native subunit conformation, which presumably reflects the delicate balance between the two modes of reversible association of the protein, which is in turn dictated by the assembly process of the virus, and its need to dissassemble completely to release its RNA. Other viruses, such as bacteriophage R17, whose life cycles may never require them to disassemble their capsids, often have intersubunit bond strengths of the same order as in TMV stacked disk rods. It is notable, also, that protein cleavage is a necessary step in the maturation of many viruses (such as bacteriophage T4 [12]), where it presumably also serves to induce conformational changes and maybe to lock the structures with tighter intersubunit bonds.

It is interesting that stacked disk rod protein contains cleavages at several different places, not just a singly highly specific site. Evidently it is physical breakage of the polypeptide chain anywhere within a particular region of the subunit which is the determining factor for the structural change involved. An unpublished compilation of available information about the course of the TMV polypeptide chain suggests that this region may be at an inner radius in the virus rod, between the central hole and the RNA binding site. Further speculation is probably best left until the detailed three-dimensional structure of TMV protein is available.

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