VI. 1. THE MASKED —SH GROUP IN TOBACCO MOSAIC VIRUS PROTEIN

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I. Introduction

The tobacco mosaic virus (TMV) particle is composed of about 94% protein and 6% ribonucleic acid. The protein consists of peptide chains of an approximate molecular weight of 18,000, the virus rod representing an aggregate of these subunits. The nature of the inter-subunit bonds which through their specificity and affinity assure the architecture and the stability of the virus particle is under active study in our laboratory. It appears certain that no covalent bonds are involved. Evidence has been reported for the participation of undissociated carboxyl groups in this bonding, presumably through hydrogen bridging (*1*, *2*). There also is present in each subunit one cysteine residue, and it is this group on which we will here focus our attention.

II. Reaction of TMV with Iodine

The reaction of TMV with iodine has been repeatedly studied. A few years ago we were able to elucidate definitely the first reaction product resulting from the interaction of TMV with KI_3 at pH 7 (*3*). It was demonstrated that each —SH group reacted with one molecule of iodine and was transformed into a sulfenyliodide (—SI) group. In contrast to the great liability of small molecular sulfenyliodides, this group in the virus was found completely stable, until the virus was degraded and its protein denatured.

Thus it was concluded that the depressed reactivity of many protein

—SH groups, i.e., their "masked nature" might be transferred to those derivatives which they were able to form. Upon denaturation of the virus, the sulfenyliodide group decomposed, releasing its iodine. In the presence of an added thiol, e.g., cysteine, this was bound by the iodo-TMV through disulfide linkage upon denaturation, but not in the native state (Protein —SI + RSH → Protein —S—S—R + H^+ + I^-). The use of I^{131} was a convenient tool in these studies. The important implications of this finding of a sulfenyliodide group in a protein for (1) the mechanism of oxidations of thiol groups in general, and (2) enzyme mechanisms has been discussed (*3*).

III. Reaction of TMV with Mercurials

Another class of reactions which were studied in our laboratory was that with heavy metal derivatives. While *p*-chloromercuribenzoate (PCMB) did not react with the virus, methyl mercuric nitrate (MMN) was bound in stoichiometric manner, i.e., one for each —SH group. Lead was also bound by the virus, but this reaction was independent, i.e., non-competitive with that of MMN. It was concluded that the mercury compound formed a mercaptide with the —SH group while the lead was bound elsewhere, and evidence was adduced suggesting that this latter binding (of lead) occurred at the above mentioned carboxyl groups involved in interunit bonding (*2*).

Recent experiments have shown, in accord with expectation, that the mono-iodo virus does not bind any MMN. Upon iodination of the MM-virus, in turn, iodine consumption was quite slow (2 atoms of iodine per mole protein in 24 minutes at 24°, instead of in 6 minutes with ordinary TMV). The reaction product retained about 0.35 moles of mercury. It thus seems that the —S—$HgCH_3$ is oxidized to the —SI group with considerably lesser ease than is the original —SH group.

From the earlier studies which have here been reviewed, it was thus evident that the approximately 2700 SH groups occurring in each TMV rod did not give the nitroprusside test, did not react with iodoacetate, or PCMB, and were not susceptible to autoxidation (with copper as catalyst) or to many oxidizing agents. On the other hand they reacted rapidly and quantitatively with MMN and iodine, yielding a substitution and a substituted oxidation product. It was concluded that their H atom was free to be replaced by those reagents which could approach it sufficiently, but that there was considerable steric or configurational hindrance about this group which prevented many typical —SH reagents from interacting, and which also stabilized the normally unstable —SI group formed in the iodine reaction. The simplest explanation of this would be that the SH group was

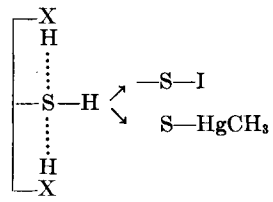

FIG. 1.

involved as the acceptor in a hydrogen-bonded structure (Fig. 1). Theoretical considerations do not support the possibility that —SH groups play a major donor role in hydrogen bonding, but the proposed acceptor position may be less objectionable. In any case, we have experimental evidence, thanks to Benesch and co-workers (*4*), that hydrogen bonding does affect the reactivity of small molecular —SH compounds, and this gives us a basis for postulating its existence and its more marked effect in the macromolecules.

IV. Role of —SH Bonding in Virus Architecture

The next question to which we have addressed ourselves experimentally in recent months is the role of the sulfur bonding in the architecture of the virus. If the reactivity of the —SH group were the same in the intact virus and in the isolated protein subunit, then one would have to conclude that the —SH groups were not involved in inter-subunit linkages. On the other hand, if the SH groups of the intact virus differed in their behavior from those of its building blocks, then their participation in such bonding would appear likely.

In order to be able to discuss this problem, we have to review in more detail the nature of the subunits involved. Complete disaggregation of the virus protein can be produced by means of anionic detergents, urea, guanidinium salts, etc. Complete disaggregation is always concomitant with complete denaturation. The product is insoluble over a wide pH range and its —SH groups are freely reactive and very autoxidizable. While a physico-chemical characterization of the material is difficult because of its tendency to aggregate and precipitate, it appears that disaggregation has here proceeded to the single peptide chain stage.

Of considerably greater interest is the material obtained by degrading the virus at low temperature with either dilute alkali (pH 10.5-11), or with 67% acetic acid. Protein so isolated, and separated from the nucleic acid behaves like a native protein. This protein, which has been termed the A-protein, is water soluble, except between pH 4 and 6. Within this

pH range, or over a wider range at higher salt concentration, its solutions become opalescent and the electron microscope reveals extensive aggregation to rod-like particles resembling the virus. While this process is largely reversible, the simultaneous aggregation of nucleic acid and protein leads to the reconstitution of virus rods which are indistinguishable from the original virus. This type of protein preparation, when not reaggregated, shows sedimentation values near 4S from which molecular weights of 90,000–120,000 have been calculated. It thus consists of 5 or 6 peptide chains, and contains that number of —SH groups. These groups are masked, i.e., they are not autoxidizable, and do not react with nitroprusside, or iodoacetate, until the protein has been denatured. It would thus seem at first sight that the reversible disaggregation of the virus into its 500 native building blocks can be achieved without affecting the nature of the —SH groups, and that these, therefore, do not participate in that aggregation process. They might nevertheless play an important structural role for the A-protein, contributing to the bonding of its 6 component peptide chains.

However, it appears possible from recent studies that the similarity in the behavior of the —SH groups in the virus and in the isolated A-protein may be more apparent than real. For, it has recently been observed that the methyl mercury group could be displaced from the protein by treatment with cysteine at pH 7.0, but that the virus mercaptide was completely stable, even if treated with 0.1 *M* cysteine for 18 hours at 25° (Table I).

TABLE I

Hg Content of MMN-Treated TMV[a]

	μM Hg/18 mg.
Intact MM-Virus (A)	0.85, 0.87
Native Protein, MMN-treated	1.02
Native Protein, prepared from A (B)	0.87
A + 0.1 *M* cysteine, pH 7.5, 18 hours, 24°	0.79, 0.81
B + 0.1 *M* cysteine, pH 7.5, 18 hours, 24°	0.00
B + 0.1 *M* cysteine, pH 7.5, 1 hour, 24°	0.08

[a] As determined by the dithizone reaction.

Another difference which was previously described is shown by the iodine reaction, for only the virus forms the stable sulfenyliodide group, while the protein —SH group becomes oxidized, probably not to a disulfide but directly to the sulfonic acid stage.

On the basis of these observations the working hypothesis has been formulated that the —SH group in the virus may actually be engaged in

the inter-A-protein bonding, but that gentle conditions of degradation permit a transfer of its bonding affinity to an internal attachment site. The resultant intra-A-protein bond would have lost the quite singular stability of the original intermolecular bond, and would be in a similar masked state as the SH groups of ovalbumin and other proteins. It was thought that evidence for this hypothesis could be sought in attempting to trap the —SH group at the moment of transition from the one state to the other. To this end the virus was degraded to A-protein by means of alkali in the presence of C^{14}-iodoacetate and the amount of the reagent bound by the protein under these conditions (*in statu nascendi*) was compared with that bound by finished (i.e., stabilized) A-protein.

The results of these experiments have not as yet been sufficiently consistent to permit any definite conclusions. It may well be that the conditions of pH and temperature required for the splitting of the virus cause the reversible denaturation of the protein, be it *in statu nascendi,* or isolated.

V. Reconstitution of Virus with MM-Protein

Another approach to the problem of the role of —SH groups in maintaining viral architecture was to study the suitability of the MM-substituted protein for reconstitution, or its ability to reform that architecture. This was studied in various ways. It was found that MM-substituted protein could be prepared from the substituted virus by splitting it with 67% acetic acid in the usual manner, without any loss of the substituent (Table I). It could also be prepared by the addition of the reagent to the isolated protein. Such MM-substituted protein preparations were then treated with viral nucleic acid and pyrophosphate buffer under favorable conditions for reconstitution (ref. *5*). The reconstitution reaction was also performed with unmodified protein in the presence of a slight excess of the reagent. The results were the same in all experiments. An appreciable fraction (30–40%) of the substituted protein precipitated out of the reaction mixture, be it that its solubility in 0.1 *M* pyrophosphate is limited to 0.05%, or, more probably, that this solvent differentiates native from slightly denatured mercury protein. The rest of the protein combined with the nucleic acid, though with lesser efficiency than did the unsubstituted protein. The sedimentable fraction of the reaction product had about 4% of the infectivity of TMV, while controls with mercury-free protein showed about 30% of the infectivity of TMV. One may conclude from these experiments that the substitution of the —SH group does not abolish the ability of the protein to aggregate to rods, but decreases the efficiency of this process.

VI. Summary

The tobacco mosaic virus protein consists of about 2,700 peptide chains each carrying one —SH group. These —SH groups show unique properties and reactivities. Thus they bind a methyl mercury residue in so stable a manner that it cannot be split off by excess thiols, and they become oxidatively substituted by iodine to a stable sulfenyliodide group. From this it is concluded that the thiol sulfur is engaged in secondary bonding while its hydrogen is free and can be substituted. Evidence is adduced that the bonding of the sulfur contributes to the forces holding the protein subunits in place and thus assuring the architecture of the virus rod.

References

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Discussion

BENESCH: Have you brought about this S^+ oxidation by two electron oxidants, such as indophenol dyes?

FRAENKEL-CONRAT: No. Purely from laziness we have not searched for methods of obtaining the sulfenic acid group because it is more difficult to detect than the radioactive iodine which can be counted.

BENESCH: If one molecule of dye reacted only with one single —SH group, it would not be as elegant a proof as with radioactive iodine, but it would be supporting proof.

I would like to ask you about the —SH group in bovine serum albumin, the oxidation of which you investigated in collaboration with Dr. Howard Schachman. In your paper it seemed that you had not reached any definite conclusions as to whether this SH group could be oxidized to the RS^+ state. Could you say more about that?

FRAENKEL-CONRAT: The reason why we hoped to find a stable sulfenyl iodide in serum albumin was because Hughes had already described that in serum albumin one needs two atoms of iodine to oxidize the single sulfhydryl group. However, we have been unable to clarify this reaction. Since the iodine was not bound, we suspected a secondary dimerization. That is what Schachman unsuccessfully looked for. In what manner the mechanism of oxidation of the serum albumin differs from that of TMV, we do not know.

BOYER: I would say what has become a familiar phrase with this discussion, "I like your data, but I don't like your interpretation." This is in relation to the reactivity of the sulfhydryl group and the possibility that hydrogen bonding might pre-

vent the reaction. I wonder if this is a feasible explanation because of the weakness with which sulfur will act as an acceptor for hydrogen in a hydrogen bond. Any such bonds would likely be very weak bonds and equilibria would be readily displaced if there was possibility of a mercurial approaching and thus reacting with the sulfur.

Your reference to a sandwich explanation suggests that we should not eliminate another possible culinary explanation, namely that the virus is somewhat analogous to Swiss cheese with inaccessible holes and regions. Thus the sulfhydryl may be in a certain steric region where the iodine can get at it, but the mercury cannot. Further the nucleic acid might make this into a worse situation by plugging up the hole in this Swiss cheese. I am not sure the steric explanation is the real explanation, but I feel that the hydrogen bond would be an unlikely explanation.

WALLENFELS: The astonishing properties of this sulfenyliodide-virus resemble in some respects, the properties of the aromatic sulfenyl chlorides like dinitrophenyl-sulfenylchloride which is polarized to $R—S^+$ and Cl^-. For the chemical basis of this masking action one would expect an electron-attracting environment in the virus protein. Perhaps, as Dr. Boyer pointed out, some part of the protein can extract electrons from sulfur, endowing it with properties like those of aromatic sulfenyl chlorides.

FRAENKEL-CONRAT: I should have said that the properties of the sulfenyl iodide virus are unusual for an aliphatic sulfenyl iodide. But the protein structure makes it act differently from cysteine sulfenic acid.

LINDLEY: I should like to point out that the reagents which react with the thiol group of the virus seem to be soluble in nonpolar solvents whereas the unreactive reagents are water soluble. When Rollett and I built the model of insulin (*Biochim. et Biophys. Acta,* **18**, 183, 1955) we were very impressed by the fact that the space between the two α-helices was filled with hydrocarbon side chains. If one imagines the thiol group of the virus in the middle of such an environment, I think it is quite likely that water soluble reagents would never penetrate to react with the thiol group.

FRAENKEL-CONRAT: It would still require spatial limitation, because I think PCMB is less water soluble or more nonpolar than methyl mercury and yet, PCMB does not get at that group. But if one postulates Boyer's cheese together with lots of leucine forming the inner wall of the hole in the cheese, then one might arrive at an explanation.

BOYER: I guess I am not on record as being an analyst but Swiss cheese sometimes has considerable fat in it, although it is lower than in most cheese.

HAUROWITZ: I would like to ask this. When we iodinate proteins, the protein acquires a yellowish color; one can remove most of this color by thiosulfate, but not all of it. It was suggested a few years ago that periodide, I_3^-, is formed on, I think, the imidazole group of histidine. Are these sulfenyl iodides yellowish?

FRAENKEL-CONRAT: They are colored, yes. When we perform the reaction and then ultracentrifuge the virus then the pellet is distinctly yellow. I did not mention that when we put mercury on the sulfur, then the iodine does not react with the sulfur and the product is not yellow. So we definitely can, by looking at it, already differentiate the sulfenyliodo virus from other reaction products or from untreated virus. I forgot to mention that we looked hard for other proteins showing a similar iodine (I^{131}) binding which was reversible by denaturation. This was the criterion; iodine introduced into a protein which is released upon denaturation. However, we found no iodine binding of that nature in any other sulfhydryl or non-sulfhydryl protein.

HAUROWITZ: It is difficult to believe in stable sulfenyl iodides. We thought it

might be perhaps clathrate formation and the iodine might be present as an occlusion compound. I wonder if you considered this possibility. This seems more probable than sulfenyl iodides.

FRAENKEL-CONRAT: With TMV we get no oxidation of the SH to a disulfide group. In other proteins you find the amount of iodine consumed to be equivalent to the SH ($2SH + I_2 = —S—S— + 2HI$). With TMV in contrast we need two equivalents of iodine for the sulfhydryl to disappear. In this protein, and only in this protein, do we find the yellow color and the iodine bound until the protein is denatured, and so on. I think it would be difficult to think of any simple artifact which would not be occurring also in other proteins.

WALLENFELS: Do you know if it reacts only with cysteine or also with other amino acids?

FRAENKEL-CONRAT: In the literature we find that sulfenyl iodides react with amino groups. We have tried to trap it in the presence of lysine and see if we could introduce lysine and that was unsuccessful.

WALLENFELS: They react with SH groups.

FRAENKEL-CONRAT: Yes, but we were unable to prove that it reacted with amino groups, but it might be a question of conditions.

BROWN: I would like to make one point that Dr. Wallenfels touched on. Kharasch worked with many aromatic sulfenic acids as well as with sulfenyl chlorides. With such aromatic compounds with very positive sulfur atoms there is much more stability than with aliphatic compounds where such derivatives are unstable. It still has to be explained why this particular protein polypeptide chain should confer particular properties on this particular SH group.

JENSEN: You can store aliphatic sulfenyl chlorides provided you keep them cold because these have been made as intermediates in the synthesis of thiol phosphate esters from mercaptans. You can't let them stand at high temperatures; they are delicate substances.

BENESCH: It is only the sulfenic *acids* which are unstable. The halides are more stable, and the sulfenamides are quite remarkably stable.