The Reactivity of Functional Groups as a Probe for Investigating the Topography of Tobacco Mosaic Virus

THE USE OF MUTANTS WITH ADDITIONAL LYSINE RESIDUES IN THE COAT PROTEIN

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Several mutants of tobacco mosaic virus that contain additional lysine residues as a result of mutations in the coat protein were investigated. Mutant E66 has a lysine residue replacing asparagine at position 140 when compared with the wild-type *vulgare* and this lysine residue reacts readily in the intact virus with methyl picolinimide. Mutant B13*a* has two new lysine residues in the coat protein, replacing a glutamine at position 9 and an asparagine at position 33, whereas mutant B13*b* has the single replacement of glutamine by lysine at position 9. The lysine residue at position 9 in mutants B13*a* and B13*b* also reacts readily with methyl picolinimide in the intact virus but the lysine at position 33 in mutant B13*a* did not react under these conditions. However, when the isolated coat protein from mutant B13*a* was treated with methyl picolinimide, the lysine residue at position 33 did become modified, showing that the loss in reactivity of this residue towards the imidoester in the intact virus is a result of the assembly of the protein subunit into the virus structure. These results are compatible with and extend previous studies on the serological properties of mutants of tobacco mosaic virus and illustrate the value of methyl picolinimide as a reagent for probing the accessibility of amino groups in proteins. When intact tobacco mosaic virus (*vulgare*) was treated with *p*-iodobenzenesulphonyl chloride, no reaction with the lysine residues at positions 33 or 68 in the virus subunit could be detected but complete modification of tyrosine-139 was achieved. This result also extends previous studies with other reagents. The usefulness of the differential reactivity of the lysine residues in tobacco mosaic virus and its mutants as a means of attaching heavy-atom labels at chemically defined positions for subsequent X-ray-diffraction analysis and the implications of these experiments for deciphering the folding of the peptide chain in the virus subunit are discussed.

Despite the progress made in the study of the structure of tobacco mosaic virus (TM virus) by electron microscopy and X-ray-diffraction analysis (Barrett *et al.*, 1972) it is evident that owing to the limited resolution obtainable with this system, no exact answer can yet be given to the question of the folding of the polypeptide chain in the virus protein subunit [for reviews see Caspar (1963) and Fraenkel-Conrat (1964)]. Other methods can make valuable contributions to a solution of this problem and, in particular, useful information has been derived from analysis of mutations in the coat protein. Investigations of the electrophoretic and immunological behaviour of such mutants of TM virus (von Sengbusch, 1965; van Regenmortel, 1967) can allow an informed guess as to whether the residues concerned occupy 'surface' or 'buried' positions in the intact virus and examination of the chemical reactivity of functional groups in the protein can be used similarly. Thus, of the two

ϵ -amino groups in each protein subunit of intact TM virus, that of lysine-68 reacts readily with acetic anhydride (Fraenkel-Conrat & Colloms, 1967) and maleic anhydride (King & Perham, 1971) and with a variety of imidoesters (Perham & Richards, 1968; Perham & Thomas, 1971), whereas that of lysine-53 reacts poorly if at all. Moreover, neither amino group reacts with 1-fluoro-2,4-dinitrobenzene (Anderer, 1963) or trinitrobenzenesulphonic acid (Scheele & Lauffer, 1969). Since both amino groups are capable of reaction in the isolated virus protein (Fraenkel-Conrat & Colloms, 1967; Perham & Richards, 1968), it has been proposed that in the intact virus lysine-53 has the properties of a buried residue and that lysine-68 can only react with reagents no bigger than an unsubstituted benzene ring, i.e. it is partially 'accessible' (Perham & Richards, 1968).

These unusual properties of the amino groups in the

vulgare strain of TM virus [the *N*-terminus is acetylated (Anderer *et al.*, 1965; Funatsu *et al.*, 1964)] suggested that it might be worth examining mutants of TM virus that contain additional lysine residues in the coat protein. A study of the reactivity of the amino groups should provide information, albeit circumstantial, about the location of the residues with respect to the virus surface. In addition, the ability to label selectively the amino groups in TM virus can be turned to advantage in the preparation of heavy-atom derivatives by chemical modification (King & Perham, 1971; Perham & Thomas, 1971). Such derivatives might then be used to find the exact three-dimensional position of the modified residue by X-ray-diffraction analysis (see Barrett *et al.*, 1972).

The present paper describes an examination of suitable mutants of TM virus. The locations of the lysine mutations in the primary structure have been established and the reactivity towards the imidoester, methyl picolinimide, investigated. Some attempts to prepare heavy-atom derivatives by reaction of the virus with *p*-iodobenzenesulphonyl (pipsyl) chloride are also described.

Materials and Methods

Tobacco mosaic virus and reagents

TM virus *vulgare* and the mutants B13a and E66 were a generous gift from Dr. R. Leberman, to whom the mutants had originally been given by Professor Melchers. The mutant B13b was kindly given by Dr. P. von Sengbusch. The virus A protein was prepared by the acetic acid method of Fraenkel-Conrat (1957) and stored frozen.

Trypsin (twice recrystallized) and chymotrypsin were from Worthington Biochemical Corp., Freehold, N.J., U.S.A., and *N*-ethylmorpholine was purchased from Kodak Ltd., Kirkby, Liverpool, U.K. Iodoacetic acid (British Drug Houses Ltd., Poole, Dorset, U.K.) was recrystallized from heptane before use. All other reagents were of A.R. grade. Methyl picolinimide was synthesized as described by Benisek & Richards (1968).

Reaction of TM virus with methyl picolinimide

The intact virus or A protein (about 10 mg/ml) was treated with 0.1 M-methyl picolinimide in 0.1 M-*N*-ethylmorpholine-acetic acid buffer, pH 8.2, for the prescribed length of time at room temperature (20°C). The changed virus or protein was purified by dialysis against 5 mM-*N*-ethylmorpholine-acetic acid buffer, pH 8.2, at 2°C and the extent of reaction with protein amino groups was measured at 262 nm in a Unicam SP.500 spectrophotometer (Perham & Richards, 1968; Benisek & Richards, 1968).

Peptide 'maps'

Proteins were *S*-carboxymethylated and digested with trypsin as described by Gibbons & Perham (1970). Proteolytic digestion with chymotrypsin was done under the same conditions as those used for trypsin. Peptide 'maps' were prepared as described by Perham & Richards (1968) and stained with the ninhydrin-cadmium reagent of Heilmann *et al.* (1957). Details of the conditions used for paper electrophoresis have been given by Perham (1967). Amide assignments in peptides were made from the electrophoretic mobility (*m*) of the peptide at pH 6.5 (Offord, 1966), defining the mobility of aspartic acid as -1.0.

For amino acid and *N*-terminal analysis, peptides were eluted with 0.1 M-NH₃ from peptide 'maps' lightly stained with 0.01 % (w/v) ninhydrin in acetone.

Amino acid analysis

Peptides and proteins were hydrolysed with 6 M-HCl for 24 h at 105°C in sealed, evacuated tubes and analysed for amino acids by using a Beckman 120C automatic analyser as described by Perham (1967).

Identification of N-terminal residues

The *N*-terminal residues of peptides were identified by means of the dansyl technique (Gray & Hartley, 1963; Hartley, 1970). The Dns-amino acids were separated by t.l.c. on polyamide layer sheets (British Drug Houses Ltd., Poole, Dorset, U.K.) by using the solvent systems of Woods & Wang (1967).

Results

Mutant E66

The mutant E66 is a spontaneous mutant of TM virus that differs from the *vulgare* strain in having a lysine residue substituted for asparagine at position 140 (Wittman, 1964). It was treated with 0.1 M-methyl picolinimide for 15 h and then purified by dialysis against 5 mM-*N*-ethylmorpholine-acetic acid buffer, pH 8.2, at 2°C. The A protein was prepared from the changed virus, before being *S*-carboxymethylated and then digested with trypsin. Samples of *S*-carboxymethylated A protein from *vulgare* and untreated mutant E66 were also digested with trypsin and the three tryptic digests compared by peptide 'mapping'. As shown in Fig. 1, *vulgare* and mutant E66 differ in that peptide VT4 of *vulgare* is replaced by peptides E66T4a, E66T4b and E66T4c. The last-named may be identified as free arginine from its position in the peptide 'map'. Amino acid and *N*-terminal analysis of the various peptides (Table 1) is sufficient to show that, given the amino acid sequence of *vulgare* protein (Anderer *et al.*, 1965; Funatsu *et al.*, 1964),

Fig. 1. Peptide ‘maps’ of tryptic digests of *S*-carboxymethylated coat proteins from (a) *vulgaris*, (b) mutant *E66* and (c) mutant *E66* that had been treated with methyl picolinimide

Electrophoresis was at pH6.5, followed by chromatography in butan-1-ol–acetic acid–water–pyridine (15:3:12:10, by vol.). ↓, Origin.

Table 1. Amino acid compositions and inferred sequences of tryptic peptides from the coat protein of *TM virus vulgaris* and mutant *E66*

For experimental details see the text.

Amino acid	Amino acid composition (mol/mol of peptide)						
	E66T4a	E66T4b	VT4	E66T1	E66T2	E66Tx1	E66Tx2
Lys	1.0	1.0		1.0		0.6	0.4
Arg		1.1	0.9		1.0	1.0	1.1
Asp			1.1	2.1		2.2	
Thr	1.0	1.0	0.9				0.9
Ser	0.9	0.8	0.8	0.9		0.8	0.8
Pro				1.0		1.0	
Gly	1.8*	1.9*	2.0*				2.1*
Val					0.8*	0.8	
Tyr	0.7	0.7	0.7		0.7	0.7	0.8
Phe				1.8*		1.8*	
Peptide	Inferred sequence						
E66T4a	Gly-Thr-Gly-Ser-Tyr-Lys						
E66T4b	Gly-Thr-Gly-Ser-Tyr-Lys-Arg						
VT4	Gly-Thr-Gly-Ser-Tyr-Asn-Arg						
E66T1	Phe-Pro-Asp-Ser-Asp-Phe-Lys						
E66T2	Val-Tyr-Arg						
E66Tx1	Phe-Pro-Asp-Ser-Asp-Phe-Lys†-Val-Tyr-Arg						
E66Tx2	Gly-Thr-Gly-Ser-Tyr-Lys†-Arg						

* *N*-Terminal residue established by the dansyl procedure.
† Modified lysine residue formed by reaction of the ε-amino group with methyl picolinimide.

peptide VT4 has the sequence Gly-Thr-Gly-Ser-Tyr-Asn-Arg and peptides E66T4a and E66T4b are Gly-Thr-Gly-Ser-Tyr-Lys and Gly-Thr-Gly-Ser-Tyr-Lys-Arg respectively. The products of tryptic digestion are therefore compatible with the substitution of lysine for asparagine at position 140 in the coat protein as proposed for mutant E66 by Wittmann (1964).

On the other hand, the peptide 'map' of the tryptic digest of coat protein prepared from mutant E66 after its treatment with methyl picolinimide (Fig. 1c) shows the disappearance of peptides E66T1, E66T2, E66T4a, E66T4b and E66T4c and the appearance of two new peptides E66Tx1 and E66Tx2. Amino acid and end-group analysis (Table 1) is sufficient to show that E66Tx1 is the 'bridge' peptide to be expected in a tryptic digest if lysine-68 reacts with the imidoester and that E66Tx2 is the 'bridge' peptide to be expected if lysine-140 also reacts. Since spectrophotometric analysis indicated a total of 2.1 picolinimidyl groups bound per molecule of E66 protein and the peptide 'maps' showed the modification of lysine-68 and lysine-140 to be complete, it is clear that lysine-53 has remained essentially unmodified by reaction with the imidoester. [As explained by Perham & Richards (1968), modification of lysine-53 with imidoesters is difficult to detect by direct peptide 'mapping' techniques because the lysine-53-proline-54 bond is resistant to trypsin even when the lysine residue is unmodified. However, this indirect method serves very well.]

Not surprisingly, lysine-53 in the mutant E66 exhibits the same lack of reactivity as the corresponding residue in *vulgare*. The lysine residue at position 140, however, is at least as reactive as lysine-68. In experi-

ments that involved shorter times of reaction of virus with imidoester, peptide 'mapping' revealed that it was impossible to modify lysine-68 without simultaneously modifying lysine-140. By this criterion, therefore, lysine-140 displays the reactivity towards methyl picolinimide normally associated with the ϵ -amino groups of proteins (Perham & Richards, 1968; Benisek & Richards, 1968).

Mutant B13

The mutant B13 is a spontaneous mutant of TM virus which differs from *vulgare* in having lysine substituted for asparagine at position 33 (Wittmann & Wittmann-Liebold, 1966). A sample from the preparation B13a was treated with 0.1M-methyl picolinimide for 15h and the A protein then prepared and *S*-carboxymethylated. Samples of A protein from *vulgare* and untreated mutant B13a were also *S*-carboxymethylated and peptide 'maps' of the tryptic digest of all three proteins were compared. As shown in Fig. 2, the 'map' of mutant B13a differs from that of *vulgare* by the presence of two additional tryptic peptides, B13aT5 and B13aT6. The amino acid and *N*-terminal analyses of these peptides (Table 2) are sufficient to identify them as residues 1-9 and 34-41 respectively in mutant B13a. The electrophoretic mobilities at pH6.5 are compatible with the amide assignments given. The presence of the peptides can be explained by an unexpected lysine/glutamine substitution at position 9 and the expected lysine/asparagine substitution at position 33 in the B13a coat protein. In peptide 'maps' of *vulgare*, the *N*-terminal tryptic peptide (residues 1-41) remains at the origin (Perham & Richards, 1968).

Fig. 2. Peptide 'maps' of the tryptic digest of *S*-carboxymethylated coat protein from mutant B13a

(a) is a 'map' of the coat-protein digest and (b) is a peptide 'map' of a tryptic digest of the *S*-carboxymethylated coat protein from mutant B13a obtained after treatment of the isolated coat protein with methyl picolinimide. Conditions of electrophoresis and chromatography are those of Fig. 1. $\uparrow$, Origin.

Table 2. Amino acid compositions and inferred sequences of tryptic and chymotryptic peptides from the coat protein of TM virus mutants B13a and B13b

Amino acid	Amino acid composition (mol/mol of peptide)					
	B13aT5	B13aT6	B13aC1	B13aC2	B13bC1	B13bC2
Lys	1.0		1.0	1.0	1.0	
Arg		1.0				
Asp						1.1
Thr	2.2	0.9	1.9		1.8	
Ser	2.8		1.6*		1.6*	
Glu		4.3*		1.2		1.1
Pro	1.1		0.8		1.0	
Ala		1.1		0.7*		0.6*
Gly				1.2		1.2
Leu				1.1		1.0
Ile	1.1		1.0		0.9	
Tyr	0.9					
Phe		1.0	1.0	0.9	1.1	1.1
Peptide	Inferred sequence					
B13aT5	Ac-Ser-Tyr-Ser-Ile-Thr-Thr-Pro-Ser-Lys					
B13aT6	Gln-Phe-Gln-Thr-Gln-Gln-Ala-Arg					
B13aC1	Ser-Ile-Thr-Thr-Pro-Ser-Lys-Phe					
B13aC2	Ala-Leu-Gly-Lys-Gln-Phe					
B13bC1	Ser-Ile-Thr-Thr-Pro-Ser-Lys-Phe					
B13bC2	Ala-Leu-Gly-Asn-Gln-Phe					

* N-Terminal residue established by the dansyl procedure.

Since no additional peptides other than B13aT5 and B13aT6 were observed in the peptide 'maps' of the tryptic digest of B13a it seems clear that the ninhydrin-staining material remaining at the origin in this 'map' comprises residues 10–33.

These conclusions were confirmed by examining peptide 'maps' of chymotryptic digests of *S*-carboxymethylated coat protein from *vulgare* and mutant B13a. Two peptides, B13aC1 and B13aC2, changed their positions in comparison with their counterparts in the 'map' of *vulgare*. Amino acid and *N*-terminal analysis (Table 2) shows them to be residues 3–10 and 30–35 respectively, confirming the presence of lysine at positions 9 and 33. The electrophoretic mobilities at pH 6.5 are compatible with the amide assignments given. The presence of the two additional lysine residues in the B13a coat protein was also detected by simple amino acid analysis of the *S*-carboxymethylated derivative (Table 3).

When the virus B13a is treated with methyl picolinimide, peptides B13aT5, B13aT1 and B13aT2 disappear from the tryptic peptide 'maps' and are replaced by the single peptide B13aTx1. It is deduced from their characteristic positions in the peptide 'map' that peptide B13aTx1 is the 'bridge' peptide formed from peptides B13aT1 and B13aT2, corre-

sponding with modification of lysine-68 in the reaction of virus with imidoester. The disappearance of peptide B13aT5 is readily attributed to simultaneous modification of lysine-9, residues 1–33 now remaining as a single peptide at the origin of the 'map', since the continued presence of peptide B13aT6 indicates that lysine-33 cannot have been modified to any great extent. The suspected lack of reaction at lysine-33 and lysine-53 was confirmed by the observation that a total of 2.2 picolinimidyl groups were bound per molecule of B13a coat protein, assayed spectrophotometrically.

In another set of experiments, the A protein from mutant B13a was also treated with 0.1M-methyl picolinimide for 15 h and then *S*-carboxymethylated and digested with trypsin. A peptide 'map' of the tryptic digest (Fig. 2) showed that peptides B13aT5 and B13aT6 had both completely disappeared and that peptides B13aT1 and B13aT2 were replaced by peptide B13aTx1. This result indicates that lysine residues 9, 33 and 68 have now been modified by reaction with the imidoester, the peptide comprising residues 1–41 remaining at the origin of the peptide 'map', and confirms that the lysine residues are available for reaction with methyl picolinimide in the isolated A protein. Direct spectrophotometric assay

Table 3. *Amino acid compositions of the S-carboxymethylated coat proteins of TM virus vulgare and mutant B13a*

Amino acid	Amino acid composition (mol/mol of protein)			
	B13a		<i>vulgare</i> (from sequence)	Difference (B13a– <i>vulgare</i>)
	Found	Assumed integral value		
Lys	4.08	4	2	+2
Arg	11.0	11	11	0
CmCys	0.8	1	1	0
Asp	16.8	17	18	–1
Thr*	15.7	16	16	0
Ser*	15.5	16	16	0
Glu	14.7	15	16	–1
Pro	7.9	8	8	0
Gly	6.1	6	6	0
Ala	13.6	14	14	0
Val†	13.4	14	14	0
Ile†	8.0	9	9	0
Leu	11.8	12	12	0
Tyr	4.0	4	4	0
Phe	7.6	8	8	0

* Corrected for destruction during the 24 h acid hydrolysis (Perham, 1967).

† Incompletely released during the 24 h hydrolysis.

of the number of picolinimidyl groups bound was impossible because the treated A protein tended to precipitate and the simultaneous modification of lysine-53 is inferred from the accessibility of the ϵ -amino groups of the two lysine residues in the A protein subunit of *vulgare* (Perham & Richards, 1968).

When this whole series of experiments was repeated with another preparation (B13b) of the mutant, peptide B13aT6 was unexpectedly absent from a peptide 'map' of the tryptic digest of the *S*-carboxymethylated coat protein, although peptide B13aT5 remained (cf. Fig. 2). In all other respects the 'maps' of B13a and B13b coat proteins were identical. This observation suggested that the lysine/asparagine replacement at position 33 in this mutant might not have taken place, a result which was confirmed by examining peptide 'maps' of the chymotryptic digests of *S*-carboxymethylated coat protein from mutants B13a and B13b. A chymotryptic peptide B13bC1 was isolated corresponding to peptide B13aC1 but a new peptide B13bC2 was observed to replace peptide B13aC2. Amino acid and *N*-terminal analysis was sufficient to identify peptides B13bC1 and B13bC2 as residues 3–10 and 30–35 respectively (Table 2), substantiating the expected presence of lysine at position 9 and asparagine at position 33.

As expected, when mutant B13b was treated with 0.1 M-methyl picolinimidate for 15 h and examined

by the peptide 'mapping' and other techniques described above, the lysine residues at positions 9 and 68 were shown to react completely whereas that at position 53 was unmodified.

Reaction of vulgare with pipsyl chloride

As pipsyl chloride reacts with protein amino groups (Keston *et al.*, 1946, 1949) it seemed reasonable to suppose that it might be possible selectively to modify lysine-68 by treatment of *vulgare* with pipsyl chloride, thereby introducing an iodine atom that could be located in three dimensions by X-ray-diffraction analysis. The virus (20 mg in 2 ml of 0.1 M-*N*-ethylmorpholine–acetic acid buffer, pH 8.2) was treated with 20 mg of pipsyl chloride dissolved in 0.10 ml of acetone. The reaction mixture was stirred gently at 20°C for 24 h and the precipitate of undissolved pipsyl chloride was removed by centrifugation. Two further additions of fresh pipsyl chloride were made at 24 h intervals in an identical way and the virus was then purified by dialysis against water. The A protein was prepared, *S*-carboxymethylated and digested with trypsin. Examination of peptide 'maps' of the tryptic digest disclosed that little or no modification of lysine-68 (or lysine-53) had taken place but that peptide VT4 (see Fig. 1) now ran more slowly on electrophoresis at pH 6.5 and faster in the chromatographic

system. Moreover, it no longer gave the characteristic staining reaction for peptides containing tyrosine (Acher & Crocker, 1952). Since peptide VT4 has the sequence Gly-Thr-Gly-Ser-Tyr-Asn-Arg (residues 135-141, Table 1), these facts are best explained by assuming that pipsyl chloride reacts with the phenolic hydroxyl group of tyrosine-139, the reaction apparently being complete as judged from the peptide 'maps'. No other alterations to the peptide 'maps' were observed.

Discussion

The results of the present investigation show that treatment with methyl picolinimidate is a simple and convenient method for determining the 'accessibility' of amino groups in proteins. The reagent is freely miscible with water, the reaction is specific for amino groups, the extent of modification can easily be determined spectrophotometrically and the sites of modification established by peptide 'mapping' procedures (Benisek & Richards, 1968; Perham & Richards, 1968). Moreover, the fact that modification of amino groups proceeds without loss of positive charge at the side chain minimizes the risk of conformational changes that derive from changed ionic interactions.

With the mutant E66, the lysine residue at position 140 reacts readily with the imidoester, suggesting that it occupies a position at or close to the 'surface' of the intact virus. This is nicely compatible with the X-ray-diffraction analysis of mutant Ni2068 in which tyrosine-139 is replaced by a cysteine residue, the results of which place residue 139 at a radial distance of about 7.2 nm from the axis of the particle, i.e. close to the outer surface of the virus (Barrett *et al.*, 1972). However, knowledge of the three-dimensional position of residue 139 makes X-ray-diffraction analysis of mutant E66 in which lysine-140 has been suitably modified less rewarding.

The results with mutants B13a and B13b are potentially more useful [it is clear that neither of these mutants is the same as the B13 mutant originally described by Wittmann & Wittmann-Liebold (1966)]. Mutant B13a contains two new lysine residues, one of which (position 9) reacts readily with methyl picolinimidate whereas the other (position 33) must be classified as 'inaccessible'. In mutant B13b the single new lysine residue (at position 9) also reacts readily with the imidoester. The simplest interpretation of these results is that the lysine residue at position 9 is located at or near the virus surface but that residue 33 is unavailable for reaction, either because it is buried or because its side chain is engaged in some interaction that prevents reaction. The fact that lysine-33 in mutant B13a can be readily modified in the isolated coat protein indicates that the loss of reactivity is almost certainly a function of the new environment

of this residue in the assembled virus structure, a result similar to that already obtained for lysine-53 (Fraenkel-Conrat & Colloms, 1967; Perham & Richards, 1968). This finding is consistent with the observation that the replacement of asparagine by serine at position 33 in another mutant of TM virus cannot be detected serologically (van Regenmortel, 1967).

The ability to modify lysine-9 in mutant B13a or B13b should now make it possible to determine the position of this residue in three dimensions and thereby provide an important new marker for deciphering the folding of the polypeptide chain in the coat protein. The fact that lysine-9 reacts readily with methyl picolinimidate indicates that this region of the polypeptide chain probably lies near the surface of the virus particle. This would be in keeping with the hydrophilic nature of the *N*-terminal region of the polypeptide chain suggested by the preponderance of hydroxyamino acids in this part of the primary structure. The abrupt change to a more hydrophobic sequence of amino acids at residue 10, together with the observations that cysteine-27 is located at a radial distance of 5.6 nm (Barrett *et al.*, 1972) and that mutations at positions 20, 21, 25 and 33 are undetectable serologically (von Sengbusch, 1965; van Regenmortel, 1967), suggests that this region is in general more shielded from the solvent. The lack of reactivity of lysine-33 in mutant B13a is compatible with this idea.

To find one buried lysine residue in a protein is unusual enough (Perham & Richards, 1968). To find that of the four lysine residues in B13a coat protein two react poorly if at all with methyl picolinimidate in the intact virus, deserves special mention. The enforced location of a charged group in an interior position of a protein normally implies that it is accompanied by a counter-ion of opposite charge, as in the active site of chymotrypsin (Sigler *et al.*, 1968; Freer *et al.*, 1970). Unfortunately it is impossible at present to identify these counter-ions for lysine-53 or lysine-33, supposing them to exist, but most probably they are side chains of aspartic or glutamic acid residues. The presence of a new charged residue in the coat protein of mutant B13a to compensate for lysine-33 was not detected in the peptide 'maps' but it cannot be excluded on this evidence alone and a more rigorous examination of the primary structure needs to be made. Although the predominant interaction between the protein subunits of TM virus is hydrophobic in nature (Laufer & Stevens, 1968), it is clear that certain ionic interactions involving amino groups (Perham & Richards, 1968) and paired carboxyl groups (Durham & Klug, 1971; Klug, 1972) play critical roles in directing the assembly of the virus. A reliable three-dimensional model of TM virus may be necessary before these interactions can be elaborated in molecular detail but

they will provide necessary clues in interpreting the structure of the virus particle. Accumulated knowledge about TM virus mutants will be no less important.

A deficiency of the usual analysis of functional groups in proteins as being 'accessible' or 'inaccessible' to modifying reagents is that a variety of causes can render a group unreactive (Cohen, 1970; Perham & Anderson, 1970; Stark, 1970). Similarly, to say that a group displays normal reactivity and therefore occupies a surface position in the molecule is limited in value by the extent to which we understand what constitutes the surface of a protein molecule. By this token, in the case of TM virus, lysine residues 9 and 68 could equally well be lining the central hole in the virus, i.e. at about 2.5 nm radius or situated at or near the outer virus surface at a radius of anything from 7.0 nm to 9.0 nm (see Barrett *et al.*, 1972). X-ray-diffraction analysis of TM virus in which these residues are modified suitably to carry unique heavy-atom labels (King & Perham, 1971; Perham & Thomas, 1971) should resolve this problem (R. N. Perham, E. Mandelkow & K. C. Holmes, unpublished work). It may also throw some light on the lack of reactivity of lysine-68 towards 1-fluoro-2,4-dinitrobenzene, another example of the general fact that the reactivity of a functional group in proteins depends, among other things, on the reagent used to test it (Perham & Anderson, 1970).

Finally, although the attempt to label lysine-68 in *vulgare* by using pipsyl chloride was unsuccessful, the observation that complete reaction with tyrosine-139 occurred is compatible with the report (Fraenkel-Conrat & Sherwood, 1967) that tyrosine-139 is the only residue that can be iodinated in the intact virus, and with the radial distance of this residue (7.2 nm) established in the mutant Ni2068 (Barrett *et al.*, 1972). Moreover, reagents better and more specific than pipsyl chloride are now available for introducing heavy-atom labels at protein amino groups (King & Perham, 1971; Perham & Thomas, 1971; Riley & Perham, 1973).

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