Chemical Accessibility of Tyrosyl and Lysyl Residues in Turnip Yellow Mosaic Virus Capsids†

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ABSTRACT: The chemical accessibility of tyrosyl residues in TYMV capsids was studied by spectrophotometric titration and with the nitrating agent tetranitromethane. That of the lysyl residues was probed with trinitrobenzenesulfonate. Attempts to test their accessibility in virions were also made. Since some of these reactions were accompanied by structural changes, degradation of the particles were monitored with ultracentrifugation and light-scattering measurements. Alkaline titration of TYMV capsids induced ionization of two of the three tyrosyl residues per subunit at pH 11.3, but the third tyrosyl ionized with an apparent pK of 12.65, concomitantly with the degradation of the capsids. Reaction with tetranitromethane suggested that one tyrosyl residue per subunit can easily be nitrated and initiates degradation, after which the remaining residues also react. In intact capsids, five out of seven lysyl residues per subunit reacted readily with trinitrobenzenesulfonate. The other two lysyl residues were trinitrophenylated only after degradation of the capsids. On the other hand, all seven lysyl residues per subunit were easily trinitrophenylated in virions, during which reaction the virions disintegrated. The demonstrated chemical inaccessibility of specific numbers of tyrosyl and lysyl residues in TYMV capsids and the observed structural consequences to the capsids when the residues were made to react are consistent with previously published properties of the cysteinyl and tryptophanyl residues. The findings suggest that in the capsid the central region of the TYMV polypeptide chain is buried and might represent a site of contact between neighboring subunits.

Studies of the chemical accessibility of amino acid residues in viral proteins can provide clues to the disposition and possible structural function of these amino acids within the tertiary and quaternary structure of virus particles. In turn, these clues may clarify the nature of the stabilizing interactions in viral nucleoproteins and the important properties of their dissociation and reassembly.

The chemical reactivity of amino acid residues has been studied with the protein of tobacco mosaic virus (TMV)[1], and several of its natural and chemically induced mutants (for a review, cf. Kaper, 1976). These studies provided insight into the chemical topography of the particle. Also, the reactivity of the sole cysteinyl residue of TMV protein made it possible to prepare an isomorphously substituted heavy metal derivative (Fraenkel-Conrat, 1959), that led to the resolution of the quaternary structure of TMV by X-ray diffraction (Franklin et al., 1959).

With the protein of turnip yellow mosaic virus (TYMV), we are only at the beginning of this road, even though TYMV can be counted among the physically and chemically best characterized viruses (Kaper, 1968), and has served as the architectural prototype of small viruses whose protein subunits are arranged on an icosahedral surface lattice with $T = 3$ (Klug et al., 1966; Finch and Klug, 1966).

As noted in a preceding paper of this series (Kaper, 1971), from the standpoint of structural stabilization TYMV is of unusual interest. For example, its capsid is more stable than the virion under many conditions, which suggests that protein–protein linkages predominate in the structure. In addition, circumstantial evidence is mounting that the capsid stabilization is mainly hydrophobic (Jonard, 1972).

In early studies of the chemical accessibility of specific types of amino acid residues in TYMV, the cysteinyl residues were found to be unreactive with the sulfhydryl reagent 5,5′-dithiobis(2-nitrobenzoic acid), unless the virus was first degraded (Kaper and Jenifer, 1965). In intact capsids of TYMV, one of the four cysteinyl residues of the TYMV-protein subunit proved to be more reactive with *p*-mercuribenzoate (PMB) than the three others. The reaction was followed by degradation of the capsids (Kaper and Houwing, 1962a,b). This suggested that the cysteinyl residues of TYMV occupied structurally strategic positions in the capsid, and that probably the delayed reaction of three sulfhydryl groups was caused by an initial perturbation of the capsid during reaction of the first sulfhydryl, causing greater accessibility and degradation at later stages.

The notion of differential chemical accessibility of sulfhydryl groups in TYMV seems to be supported by the recently reported amino acid sequence of TYMV-protein (Peter et al., 1972). This sequence shows that three cysteinyl residues, 94, 117, and 132, are located in a large central section of the molecule (comprising residues 73–134) in which acidic and basic amino acid residues are scarce (only lysyl-109 and -131 can be found here). In contrast, the fourth cysteinyl-169 is only 20 amino acid residues removed from the C-terminus but also in a small region devoid of charged amino acid residues (positions 152–185). Moreover, the central section 73–134 also contains the two tryptophanyl residues 75 and 95, which have been found to be inoxidizable by *N*-bromosuccinimide (Stehelin, 1972).

The coincidence of a relative shortage of polar amino

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[1] Abbreviations used are: TMV, tobacco mosaic virus; TYMV, turnip yellow mosaic virus; PMB, *p*-mercuribenzoate; $C(NO_2)_4$, tetranitromethane.

acid residues in a large section of the polypeptide chain with the relative inaccessibility of three cysteinyl and two tryptophanyl residues in the same region prompted us to probe the accessibility of the tyrosyl and lysyl residues in TYMV capsids, some of which are contained in the same region. This report shows that two of the three tyrosyls are reasonably accessible to titration, whereas the third becomes available only upon degradation of the capsid. However, on reaction with tetranitromethane ($C(NO_2)_4$), the capsid degrades after nitration of only one tyrosyl residue. Also, five of the seven lysyl residues are shown to react easily with trinitrobenzenesulfonate, whereas the remaining residues can be found only in the degraded capsid. These findings are entirely consistent with a location of several unreactive residues in the apparent "apolar" middle region of the TYMV-protein subunit.

Experimental Procedure

Materials. TYMV was isolated from infected Chinese cabbage plants that had grown in an artificially lighted room at 20°C for 21 days after inoculation. The virus was purified by the method of Dunn and Hitchborn (1965). Natural capsids were removed from purified virus preparations by zonal ultracentrifugation in an equivolumetric 10–30% sucrose gradient (Van der Zeijst and Bult, 1972) that contained 0.02 *M* sodium phosphate buffer (pH 7.0). TYMV protein capsids were prepared by alkaline degradation of the virus (Kaper, 1960, 1964). Centrifugal homogeneity of the starting preparations of TYMV and of the capsids was comparable to that shown in Figure 1 of a previous publication (Kaper, 1964). All chemicals used were of analytical or best available grade and were purchased from commercial supply houses. Commercial trinitrobenzenesulfonic acid was recrystallized from 5 *N* HCl.

Apparatus. Ultraviolet light absorption was measured with the Gilford Model 2400 recording spectrophotometer.[2] Preparative ultracentrifugation was performed with the Spinco Model L ultracentrifuge. Zonal ultracentrifugation was performed with the Beckman Model L3-50 ultracentrifuge with the Ti-14 rotor. Gradients were formed with the Beckman Model 141 high capacity gradient pump. An ISCO ultraviolet analyzer and optical unit was used for monitoring sucrose gradients after rate zonal centrifugation. pH was measured with the Radiometer Model 22 pH meter equipped with a GK 2302c combined electrode. For analytical ultracentrifugation and ultraviolet spectra, the same instruments mentioned in a previous publication (Kaper, 1971) were used.

Methods. Ionization of tyrosyl residues of TYMV capsids was measured at room temperature (about 22°C) and in the presence of 1.0 *M* KCl by the ultraviolet-difference technique. Protein capsid samples (1.6 mg/ml), previously dialyzed vs. distilled water, were titrated by the addition of an appropriate amount of KOH solution (0.05–1.0 *N*, depending on pH range desired). Optical density readings were taken at 295 nm after 3 and 60 min and compared with those of a protein capsid solution of the same concentration in 1.0 *M* KCl. Differential optical density readings at 295 nm were corrected for protein subunit concentration and reported as variations of the molar extinction coefficient ($\Delta\epsilon_{295nm}$). These were plotted against the pH of the corresponding solutions. The number of ionized tyrosyl residues was calculated by means of a differential molar extinction coefficient of 2300 (Crammer and Neuberger, 1943; Sage and Singer, 1962) corresponding to the conversion of 1 mol of tyrosine into its phenolate state.

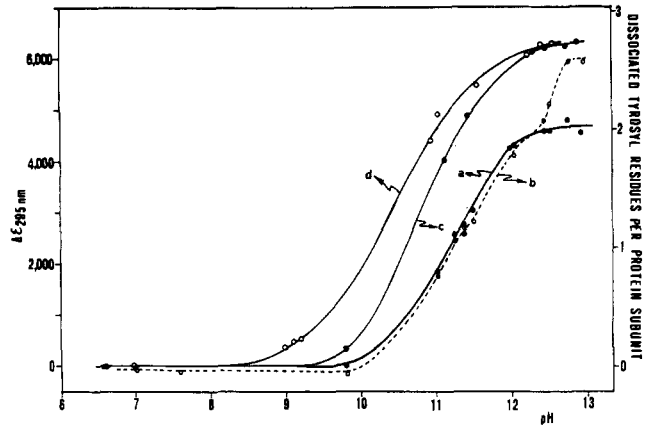

FIGURE 1: Spectrophotometric titration at 295 nm of the tyrosyl residues of TYMV capsids (1.6 mg/ml) in 1.0 *M* KCl. Variation of the molar extinction coefficient at 295 nm ($\Delta\epsilon_{295nm}$) is plotted vs. pH and is correlated with the number of dissociated tyrosyl residues per protein subunit. (a) Forward titration after 3- or 60-min exposures to the pH's indicated, without correction for light-scattering contributions. (b) 3-min forward titration corrected for light-scattering contribution at 295 nm; (c and d) back titrations after 30 min and 24-hr exposure to pH 12.5, respectively.

Light scattering was measured simultaneously with the tyrosyl ionization by monitoring the optical density at 320 nm. The $\Delta\epsilon_{320nm}$ values were converted into $\Delta\epsilon_{295nm}$ by Rayleigh's equation (cf. Schramm and Dannenberg, 1944).

$C(NO_2)_4$ reactions were carried out at room temperature (about 22°C) in a 0.05 *M* Tris-HCl buffer (pH 8.0). Capsid samples (1.75 mg/ml) were mixed with $C(NO_2)_4$ (final concentration of 0.1%, which is a 50 times excess over total tyrosyls; and which we obtained by diluting $C(NO_2)_4$ 1:10 in ethanol 95% and adding 10 μl of this solution to each milliliter of sample solution). Then, differential optical density readings were taken at 428 nm against a capsid solution of the same concentration in the same buffer without $C(NO_2)_4$. Since the reagent blank did not display significant light absorption at 428 nm, no correction of the final absorbance was needed. The number of titrated tyrosyl residues was calculated according to Sokolovsky et al. (1966) by means of a molar extinction coefficient of 4100 optical density units mol^{-1} l. cm^{-1} at 428 nm. Aliquots of the reaction mixture were withdrawn at set intervals and immediately dialyzed at 2°C against a 0.02 *M* sodium phosphate buffer (pH 7.0) for about 10 hr. Finally, the samples were analyzed in the analytical ultracentrifuge with ultraviolet optics at 280 nm.

Trinitrobenzenesulfonate reactions were carried out at room temperature (about 22°C). Virus or capsid samples (0.9 and 0.6 mg/ml, respectively) in 0.1 *M* potassium borate buffer (pH 8.0, 9.0, or 9.5, depending on the purpose of the experiment) were treated with an equal volume of 0.1% trinitrobenzenesulfonate solution (about 14 times excess over total lysyl residues) in the same buffer. Optical density readings were taken at 345 nm against a trinitrobenzenesulfonate solution of the same concentration in the same buffer. Since the light scattering at 345 nm was found to be negligible at these final concentrations of virus or capsid (0.45–0.3 mg/ml), it was not considered in the calculation

[2] Mention of a commercial company or specific equipment does not constitute its endorsement by the U.S. Department of Agriculture over similar equipment or companies not named.

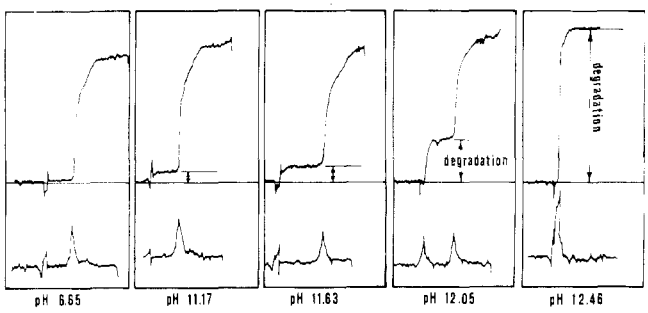

FIGURE 2: Effect of pH on the structural integrity of TYMV capsids. Photoelectric scanning patterns (280 nm) represent appropriately diluted capsid samples (1.6 mg/ml) treated for 60 min in 1.0 *M* KCl at pH 6.65, 11.17, 11.63, 12.05, and 12.46. Scans were made when the fast sedimenting boundary, if present, had assumed an approximately central position in the cell, about 8 min after centrifuge had reached the speed of 42,040 rpm. Temperature of run, 25°C.

of the results. The reaction was followed by recording the differential optical densities over an appropriate time (usually 12–18 hr). The number of trinitrophenylated ϵ-amino groups was calculated and corrected for disappearance of trinitrobenzenesulfonate, according to methods described by Goldfarb (1966) and Scheele and Lauffer (1969). At the end of the reaction, the mixtures were dialyzed overnight in the cold against a 0.02 *M* sodium phosphate buffer (pH 7.0), to eliminate excess reagent. After proper dilution, dialyzed samples were analyzed in the analytical ultracentrifuge with ultraviolet optics at 265 nm wavelength. Virus or capsid control samples were treated in the same way under the same conditions (temperature, buffer, time length, dialysis) without the reagent trinitrobenzenesulfonate.

Nitrogen was determined by the micro-Kjedahl method as used by Kaper and Litjens (1966).

Phosphorus was determined as described by Knight and Woody (1958), without further dilution, in the same virus solution that had been used for the nitrogen determination.

The protein subunit concentration of the capsid solution was calculated from its nitrogen content, by using 15.653 as the percentage nitrogen of the protein subunit [calculated from the amino acid composition and taking 20,133 daltons as the molecular weight of the subunit (Peter et al., 1972)].

The molar concentration of the protein subunits in TYMV solutions was determined from their nitrogen contents after proper adjustment for the percentage RNA of the virus, determined from the N/P ratios according to Kaper and Litjens (1966).

Results

Spectrophotometric Titration of Tyrosyl Residues in TYMV Capsids. The result of a forward titration, which is representative for different capsid preparations, is given in Figure 1 (curve a). Essentially the same S-shaped curve was obtained from the $\Delta\epsilon_{295nm}$ values at 3 and 60 min; no time dependence of the ionization was thus observed. Only two tyrosyl residues were found to dissociate between pH 9.8 and 12.9 with an apparent p*K* of 11.3, which initially suggested that the third residue might be masked even at the high pH values at which degradation of the capsid occurs (see below).

A light scattering decrease due to capsid degradation at high pH values might compensate for the hyperchromic effect resulting from the ionization of a third tyrosyl residue. Indeed, this was found to be the case at pH values 12.02, 12.46, 12.52, and 12.94, where the light absorption at 320 nm decreased with time. Ultracentrifuge analysis confirmed that above pH 12, the capsid degraded into slow-sedimenting fragments (Figure 2). At pH 11.63, the capsids were quite stable, with only a small amount of degradation showing. At pH 12.05, the capsids degraded, but the degradation was not complete even after 60 min of treatment. At pH 12.46, the degradation was complete in about 35 min, as judged from the light-scattering behavior of the corresponding solution. When the light scattering induced variations of the protein's molar extinction coefficient at 295 nm, and at different pH values, are subtracted from the forward titration curve, a corrected curve is obtained from which it can be seen that in reality 2.7 tyrosyl residues titrate. Figure 1 (dashed line) only shows the titration curve with light-scattering corrections for the 3-min treatment. The inflection at the level of two tyrosyl residues indicated a separation in the pH of ionization. It suggests that two tyrosyl residues dissociated between pH 9.8 and pH 12.3 with an apparent p*K* of 11.3, and that the third tyrosyl residue dissociated above pH 12.3 with an apparent p*K* of 12.65. (There is even some suggestion for the presence of an inflection point at pH 11.3, which could signify a separation in the pH's of ionization of the first two titrating tyrosyl residues.) Thus, in the uncorrected curve, only two tyrosyls could be found because the hyperchromicity at 295 nm due to the ionization of the third tyrosyl residue was compensated for by the hypochromicity due to the decrease in light scattering of the protein above pH 12. Since both effects are caused by degradation of the capsid, no time dependence was found in the uncorrected titration curve. If the light-scattering correction is applied after 60 min of treatment, a similar curve without inflections is obtained, probably because the capsid degradation has progressed further at the lower pH values.

Spectrophotometric back titrations were carried out after 30 min and 24 hr of alkaline treatment (pH 12.5). The results are also shown in Figure 1. Essentially, three tyrosyl residues could be titrated and showed apparent p*K*'s of 10.9 after 30 min (curve c) and 10.6 after 24 hr of treatment (curve d). The shift of p*K* (from 10.9 to 10.6) probably represented a slow unfolding of the polypeptide chain.

$C(NO_2)_4$ Reaction with Tyrosyl Residues in TYMV Capsids. Three tyrosyl residues were found to react with $C(NO_2)_4$; the kinetics of the reaction are shown in Figure 3. The reaction seemed to proceed with two phases, which is suggested by the inflection point at the level of about one residue of tyrosine. The second phase probably reflected a release of less reactive tyrosyl residues due to capsid degradation. This is shown in the sedimentation diagram for a reaction mixture sampled close to the inflection point (mixture 3), and in the amount of capsid degradation calculated from the ultraviolet sedimentation patterns plotted against the number of nitrated tyrosyl residues per subunit (inset of Figure 3).

Reaction of Trinitrobenzenesulfonate with Lysyl Residues in TYMV Capsids. At pH 8.0, 9.0, and 9.5 only five lysyl residues per protein subunit were found to react with trinitrobenzenesulfonate, both in low salt (0.1 *M* potassium borate buffer) and in the presence of 1.0 *M* KCl. Ultraviolet sedimentation patterns showed that capsids maintained their integrity after reaction with trinitrobenzenesulfonate (Figure 4a and b). However, with freeze-degraded capsids (Kaper and Alting Siberg, 1969a,b) at pH 9.5, two more groups were trinitrophenylated for a total of seven to eight lysyl residues. That total agrees with the seven expected

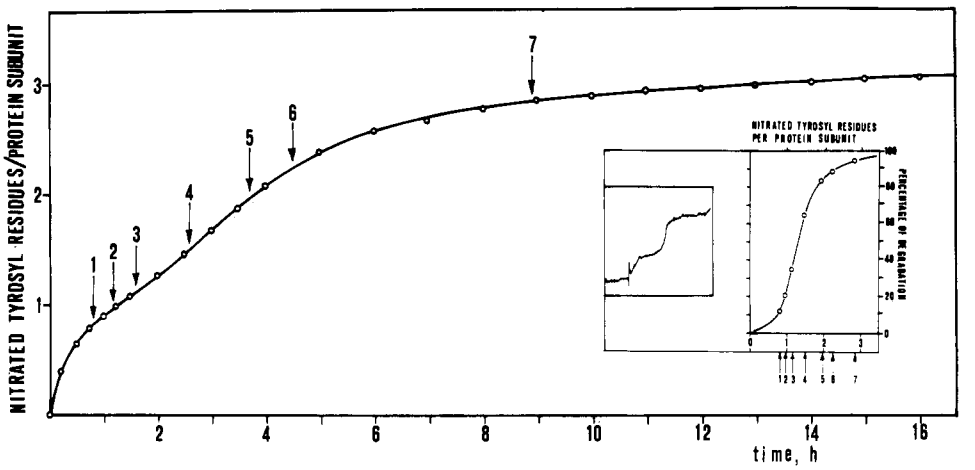

FIGURE 3: $C(NO_2)_4$ reaction with TYMV capsids (1.75 mg/ml) in 0.05 *M* Tris-HCl buffer (pH 8.0). The diagram shows the time course of the nitration of the tyrosyl residues per protein subunit. Arrows indicate the moments at which samples were withdrawn from the reaction mixture for ultracentrifugal analysis. The inset shows the percentage degradation of the capsids, calculated from ultraviolet sedimentation patterns (280 nm), plotted vs. the number of nitrated tyrosyl residues per subunit. The arrows correspond to those of the main curve, and represent the various levels of nitration. Ultracentrifugal analyses were performed after 10 hr of dialysis against 0.02 *M* sodium phosphate buffer (pH 7.0) at 2°C. An ultraviolet sedimentation pattern (280 nm) is shown, which represents sample no. 3, withdrawn during nitration of the second tyrosyl residue.

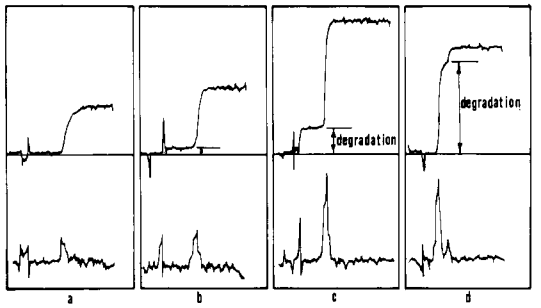

FIGURE 4: Effect of trinitrobenzenesulfonate on structural integrity of TYMV and TYMV capsids. Trinitrobenzenesulfonate reaction mixtures (pH 9.5) were dialyzed overnight in the cold against a 0.02 *M* sodium phosphate buffer of pH 7.0 and analyzed by ultracentrifugation. Photoelectric scanning patterns (265 nm) represent appropriately diluted samples of the following. (a) Untreated TYMV capsids (pH 9.5); (b) trinitrophenylated TYMV capsids (pH 9.5). Scans were made when the fast sedimenting boundary, if present, had assumed an approximately central position in the cell, about 16 min after centrifuge had reached 29,500 rpm. Temperature of run, 20°C. (c) Untreated TYMV (pH 9.5). (d) Trinitrophenylated TYMV (pH 9.5). Scans were made shortly after the centrifuge had reached 29,500 rpm. Temperature of run, 20°C.

from the amino acid composition of the TYMV protein subunit.

Preliminary Studies of the Accessibility of the Tyrosyl and Lysyl Residues in Intact Virus. Because of the high nucleic acid content of TYMV, the contribution of the RNA to the ultraviolet absorption of the virus at 295 nm is much greater than that of the protein capsid. Therefore, an optical change due to the ionization of the tyrosyl residues had to be corrected for the change in light scattering that resulted from the degradation of the virus with pH and for any effect of the pH on the absorption of the RNA (for which isolated RNA was used). Since the resulting curves were extremely complex, and uncertainty existed regarding the procedures followed, attempts to monitor the ionization of tyrosyl residues in intact viruses were abandoned to await a better understanding of the optical effects of the degradation of the virus under these conditions.

Surprisingly, seven to eight lysyl residues were found to react with trinitrobenzenesulfonate in intact TYMV. The reaction kinetics were similar to that observed for the reaction of trinitrobenzenesulfonate with freeze-degraded capsids. Ultracentrifugal analysis of the reaction mixture (Figure 4d) showed that, unlike intact capsids, TYMV that was reacted with trinitrobenzenesulfonate was almost completely degraded though the control (Figure 4c) had only released some RNA, presumably from partial alkaline degradation (Kaper, 1964).

Discussion

As suggested in the introduction, the presence of TYMV's poorly reactive cysteinyl and tryptophanyl residues in regions relatively devoid of charged amino acid residues might not be fortuitous. It could signify that these regions of the molecule could indeed be less accessible because of their possible spatial disposition in the subunit contact areas and/or at the sites of the stabilizing protein-protein interactions of the TYMV-protein capsid. Thus, a further exploration of the accessibility of particular types of amino acid residues to the solvent or to chemical modification, while monitoring the structural integrity of the capsid, should provide additional information about the molecular organization and the stabilizing interactions of TYMV.

The spectrophotometric titration of TYMV capsids revealed that the tyrosyl residues are generally less accessible to the solvent. In the intact capsid, only two tyrosyl residues per subunit could be ionized. Their apparent pK was 11.3, which is about 1.5 pH units higher than normally expected for tyrosyl residues in proteins (Tanford, 1962). However, that the ionization of these two tyrosyls took place without serious structural perturbations can be concluded, not only from the sedimentation diagrams of Figure 2, but also from the fact that a calculated titration curve of two (pK = 11.3) tyrosyl groups per subunit resembles that of the experimentally found curve (cf. Figure 1, curve a) closely. Figure 5 shows the experimental points of the corrected forward titration curve plotted according to the Linderström-Lang treatment (cf. Tanford, 1962), using the number of dissociated tyrosyl hydroxyls per subunit instead of the total charge as an arbitrary scale. The expected linearity extends only over the first two tyrosyls, after which the curve bends, indicating conformational change with titration of the third

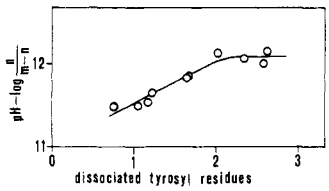

FIGURE 5: Linderström-Lang plot of the forward spectrophotometric titration of TYMV capsids (cf. Figure 1, curve b). The abcissa is an arbitrary charge scale.

tyrosyl. It seems, therefore, that the molecular microenvironment of two tyrosyl residues of each subunit is somewhat less solvent accessible, which could be due to their location in moderately hydrophobic regions of the capsid. However, both the data of Figures 2 and 4 suggest that the third residue becomes accessible to titration only upon severe structural perturbation and complete exposure of the subunit.

The amino acid sequence of TYMV-protein shows that of the three tyrosyl residues, one (tyrosyl-111) is located within the central region of the molecule, which could be tentatively designated as "apolar" (see introduction), whereas the two others (tyrosyl-66 and -147) are relatively close to this region. Thus tyrosyl-111 might represent the completely inaccessible residue, perhaps buried in the protein subunit contact areas, and is being exposed only upon degradation of the capsid, while the two others could be the ones that titrate with the unusually high pK of 11.3. The results of the back titrations, in which capsids were first exposed to extremely alkaline conditions (pH 12.5) for 30 min or 24 hr to bring about their degradation, confirm the relatively poor accessibility of the tyrosyl residues. For example, despite the considerable drop in pK (10.6 after 30 min of exposure, and 10.3 after 24 hr), which reflects the loss of the quaternary structure of the capsid, these values were still higher than expected. This probably reflects a residual tertiary structure which is progressively destroyed by prolonged alkaline treatment.

The results obtained by probing the tyrosyl residues with the nitrating agent $C(NO_2)_4$ are of considerable relevance. The biphasic character of the reaction kinetics (Figure 3) suggests that a rapid first reaction with one tyrosyl residue is followed by a temporary relaxation and a slower second phase reaction in which the two remaining tyrosyl residues are nitrated. Ultracentrifugal analysis showed that the end of the reaction of one-third of the tyrosyl residues coincided approximately with the beginning of capsid degradation. This degradation is then practically completed during the nitration of the second one-third of the tyrosyl residues. Although at first sight it may seem in conflict with the titration results discussed above, Meyers and Glazer (1971) have cited cases where it can be shown that phenolic groups in apolar locations of a polypeptide can undergo nitration or acetylation preferentially over others that may be more exposed to the solvent. In the case of TYMV capsids, the titration and nitration results could be reconciled, if during $C(NO_2)_4$ treatment the hydroxyl groups unavailable to titration would be nitrated preferentially. This process could cause structural perturbations and capsid degradation in a way that is similar to the effects observed upon the reaction of PMB with buried sulfhydryl groups in TYMV capsids (cf. Kaper and Houwing, 1962a,b).

Reaction of trinitrobenzenesulfonate with the TYMV capsid has shown that five of the seven lysyl residues per protein subunit were immediately accessible to the reagent and that their trinitrophenylation did not affect the integrity of the capsids in any obvious way. However, the two remaining lysyl residues could also be made to react upon degradation of the capsid. This observation, combined with: (1) the relative inaccessibility of the cysteinyl, the tryptophanyl, and the tyrosyl residues; (2) the demonstrated structural importance of many of these residues; and (3) the fact that most of these residues (including some lysyls) are situated within or close to the central section of the polypeptide chain, makes it attractive to speculate that in the intact TYMV capsid, this region might represent that segment of the subunit molecule that is most shielded from the solvent and from which the interprotein subunit interactions originate.

Obviously, the evidence for this hypothesis in the present and previous publications is primarily of circumstantial nature, and should be verified by direct identification of the differentially reactive amino acid residues. Our first efforts in this direction (in collaboration with the group of H. Duranton, Strasbourg, France) are under way, with an attempt to identify the sites of reaction in capsids with five lysyls per subunit trinitrophenylated.

In the reaction of trinitrobenzenesulfonate with the virions it was found that for some reason, the presence of the RNA inside the capsid caused the otherwise stable capsid structure to degrade (Figure 4d). As a result, all its seven lysyl residues were trinitrophenylated. This result can only be reasonably understood by consideration of the control reaction (Figure 4c). Although capsids are completely stable at pH 9.5, TYMV undergoes limited alkaline degradation at this pH. In separate studies of TYMV at moderately alkaline pH's (unpublished work), we have found evidence for the presence of intermediate nucleoprotein products that could represent structurally perturbed capsids in the process of releasing RNA. Perhaps, as a result of such a pH/RNA-induced capsid perturbation, the normally inaccessible lysyl residues in the intersubunit contact areas could react in part with trinitrobenzenesulfonate which, in turn, could cause further perturbation in these structurally important areas of intersubunit contacts, degrade the virion, and lead to further reaction with trinitrobenzenesulfonate.

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Selective Oxidation of Methionine Residues in Proteins†

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ABSTRACT: Methionine residues in peptides and proteins were oxidized to methionine sulfoxides by mild oxidizing reagents such as chloramine-T and *N*-chlorosuccinimide at neutral and slightly alkaline pH. With chloramine-T cysteine was also oxidized to cystine but no other amino acid was modified; with *N*-chlorosuccinimide tryptophans were oxidized as well. In peptides and denatured proteins all methionine residues were quantitatively oxidized, while in native proteins only exposed methionine residues could be modified. Extent of oxidation of methionine residues was determined by quantitative modification of the unoxidized methionine residues with cyanogen bromide (while methionine sulfoxide residues remained intact), followed by acid hydrolysis and amino acid analysis. Methionine was determined as homoserine and methionine sulfoxide was reduced back to methionine. Sites of oxidation were identified in a similar way by cleaving the unoxidized methionyl peptide bonds with cyanogen bromide, followed by quantitative end-group analysis of the new amino-terminal amino acids (by an automatic sequencer).

The thioether groups of methionine side chains in proteins are weak nucleophiles, and, like other hydrophobic residues, usually have little access to the aqueous environment. In contrast with other nucleophilic residues in proteins, they resist protonation from pH 1 to 14 and can therefore be selectively modified under acidic conditions. Alkylation with halo acids and oxidation with hydrogen peroxide were extensively used to modify methionine side chains in proteins.

Hydrogen peroxide, under suitable, relatively mild conditions, will modify several functional groups in proteins, such as thioethers, indoles, sulfhydryls, disulfides, imidazoles, and phenols. Under acidic conditions the primary reaction is oxidation of methionines (Toennies and Callan, 1939; Caldwell and Tappel, 1964), while under neutral and slightly alkaline conditions, tryptophan (Hachimori et al., 1964) and other functional groups are also modified (Means and Feeney, 1971), especially in the presence of metal ions, halide ions, organic acids, or ethers. Most studies in which a specific reaction with methionine residues has been documented have been carried out at pH below 4, such as in pancreatic ribonuclease (Neumann et al., 1962), α-chymotrypsin (Koshland et al., 1962; Schachter et al., 1963; Weiner et al., 1966), Kunitz trypsin inhibitor (Kassell, 1964), adrenocorticortropic and parathyroid hormones (Dedman et al., 1961; Tashjian et al., 1964), and chymotrypsinogen (Wasi and Hofmann, 1973). Exceptions were the oxidations of glucose oxidase at pH 5.8 (Kleppe, 1966), subtilisin at pH 8.8 (Stauffer and Etson, 1969), and bovine growth hormone at pH 8.5 (Glaser and Li, 1974).

In addition to hydrogen peroxide, a number of other reagents have been used for oxidizing methionine residues in proteins. Periodate has been shown (Clamp and Hough, 1965) to attack cysteine, cystine, methionine, tryptophan, tyrosine, histidine and N-terminal serine and threonine. It has, however, been used for selective oxidation of methionine residues in α-chymotrypsin (Knowles 1965) and apomyoglobin (Atassi, 1967) under selected critical conditions at pH 5 and 0°. Similarly azide, under acidic conditions (hydrazoic acid), was shown to oxidize methionine to methionine sulfoximine (Whitehead and Bently, 1952; Brill and Weinryb, 1967).

Iodination of proteins at neutral and slightly alkaline pH causes iodination of histidine, tyrosine, and tryptophan, as

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