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TERTIARY AND QUATERNARY STRUCTURE OF TOBACCO MOSAIC VIRUS AND PROTEIN. I. EFFECT OF pH ON FLUORESCENCE AND TNS BINDING

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SUMMARY

Tobacco mosaic virus, TMV, and its protein, TMVP, exhibit pH dependent changes in their wavelength and intensity of maximum emission, $\lambda_{max,p}$ and I_p, respectively. The binding of 2-p-toluidinylnaphthalene-6-sulfonate, TNS, to TMVP or TMV, produces a strong increase in its fluorescence intensity. This binding is pH dependent.

TMVP is severely denatured at pH > 10.5 or < 3 as indicated by large increases in $\lambda_{max,p}$. Strong increases in I_T occur concomitantly with these increases in $\lambda_{max,p}$, indicating increased binding of TNS upon denaturation. Presumably hydrophobic sites, exposed on denaturation, provide effective binding sites for TNS. Heat denaturation of TMVP at pH 7 led to similar increases in $\lambda_{max,p}$ and I_T.

In the pH range where aggregation of the protein occurs, pH 6-7, $\lambda_{max,p}$ remains constant and only minor changes in I_p and I_T occur. Major conformational changes do not appear to accompany aggregation. No significant changes in $\lambda_{max,p}$, I_p and I_T occur between pH 6 and 7 when the native virus is titrated. The changes in I_p and I_T for TMVP in this pH range are apparently associated with the aggregation of the protein.

TNS bound more weakly to the virus than to the protein at all pH values.

The self-assembly of tobacco mosaic virus protein, TMVP, has been actively investigated, as it may serve as a model system for other biological assembly processes.[1-3] The structure and properties of tobacco mosaic virus, TMV and TMVP, have been studied over a wide pH range by a number of methods such as circular dichroism,[4,5] optical rotary dispersion,[6] ultracentrifugation,[7,8] solvent perturbation,[7] hydrogen exchange,[9] and titration,[7,10] just to name a few. Having the results of these studies for comparison, it seemed worthwhile to conduct a fluorescence study of TMV and TMVP to see if any new structural information could be obtained, and to further evaluate the effects of tertiary and quaternary structure on fluorescence of proteins.

In this study we have made use of both fluorescence of the native virus and protein, and also the fluorescence of 2-p-toluidinylnaphthalene-6-sulfonate, TNS, a fluorescent probe.[11] The wavelength of maximum emission, λ_{max}, from tryptophane containing proteins is generally blue shifted from that of tryptophane in water,[12-15] but attains the same value when the protein is denatured with 8 M urea[12] or 6 M guanidine HCl.[13] This shift indicates that the tryptophane residues in the native protein are not exposed to water. TNS fluoresces weakly in water with λ_{max} near 500 nm.[11] In less polar solvents, however, λ_{max} generally decreases strongly while fluorescence intensity, I, increases strongly.[11] Similar effects are observed when small amounts of protein are added to aqueous solutions of TNS.[11] In general, proteins known to possess hydrophobic binding sites are more effective at promoting higher values of I and shorter wavelengths of emission. It was concluded that TNS binds to hydrophobic regions in proteins. Other studies with different probes have shown that electrostatic effects are also operative.[16] It has also been demonstrated[17,18]

that denaturation of proteins in the presence of 1-anilonaphthalene-6-sulfonate, a related fluorescent probe, leads to large increases in I and large decreases in λ_{max}, both effects indicating that the probe is being bound to hydrophobic regions of the protein which have been exposed by denaturation.

Experimental

Instrumental. Fluorescence measurements were carried out using a Perkin Elmer MPF-2A Hitachi spectrofluorimeter with a thermostated cell holder. Either an R-106 (blue sensitive) or an R-136 (red sensitive) photomultiplier was employed. Entrance and exit slit widths were 6 nm. In order to avoid reabsorption errors, microcells with dimensions of 3 x 3 mm were used. The protein was excited at 280 nm and fluorescence was measured at 320 nm. TNS was excited with 330 nm light, filtered through a Corning 7-54 filter and the fluorescence of TNS was measured at 440 nm. A 390 cutoff filter was placed between the sample and analyzer monochrometer. Values for I_T have been corrected for the background of unbound TNS and for the weak fluorescence from TMVP or TMV solutions in the absence of TNS. The wavelength of maximum emission from TNS + TMVP or TNS + TMV also changed with pH, but its variation was relatively small, i.e., 5 nm, and is therefore not used here.

Materials and Methods

TMV was generously donated by Dr. H. Fraenkel-Conrat. TMVP was prepared by the method of Fraenkel-Conrat.[19] A protein solution of 3 mg/ml at pH 7.5 sedimented as a single asymmetric boundary with a sedimentationn

constant of 3.7. Concentrations of virus and protein were determined spectrophotometrically, taking $E^{1\,cm}_{1\%}$ at 265 nm as 30.6 for TMV[20] and $E^{1\,cm}_{1\%}$ at 280 nm for TMVP as 12.7.[19] The absorption spectrum for TMVP at pH 7.5 had a maximum to minimum ratio of 2.3-2.5, indicating that the preparation was uncontaminated by nucleotide material.

TNS was obtained from Sigma Chemicals and was twice recrystallized from aqueous ethanol.

In carrying out the fluorescence titrations, the ionic strength was adjusted to 0.1 with NaCl. The binding of TNS was strongly increased by low concentrations of salt, but this effect leveled off at about 0.1 M. Solutions were made basic with NaOH and acid with HCl. Fresh solutions with an initial pH of 7.5 for the solutions to be titrated were used for each acid or basic titration. Measurements of $\lambda_{max,p}$ and I_p were performed in the absence of TNS. TNS was added to a fresh protein or virus solution before each acid or basic titration during which I_T was to be measured. Measurements were carried out at 20°C. As the pH of a freshly titrated solution occasionally varied with time, particularly at the pH where aggregation occurred, measurements were not taken until the pH had remained constant for 15 min. It is, however, still possible that some change in pH or fluorescence occurred after the measurements, especially at extremes of acid or base. We were not concerned with such long term changes in this study.

Results and Discussion

TMVP. The wavelength of maximum fluorescence of TMVP, $\lambda_{max,p}$, the intensity of this fluorescence maximum, I_p, and the intensity of the

maximum of TNS fluorescence, I_T, have been plotted as a function of pH and are shown in Fig. 1.

In the pH range 7-9, very little variation occurs in $\lambda_{max,p}$, I_p and I_T. Circular dichroism, CD, studies of TMVP have also shown that there are no significant changes in the mean ellipticities of any of the bands in this pH range.[4] No groups are titrated in this range,[10] and the predominant species is the trimer.[8] The fluorescence results are consistent with the other results.

In the pH range 9-10.5, a sharp decrease in I_p occurs, as well as a gradual increase in $\lambda_{max,p}$ and a small increase in I_T. Large decreases in I_p of other proteins and polypeptides in this pH range have been linked to quenching of the fluorescence of the tryptophane residues by weakly fluorescent tyrosylate groups.[21,22] It has been shown that 1-2 tyrosyl residues titrate in this region.[7] The efficiency of the quenching is quite large, ca. 75%, at pH 10.5, somewhat more efficient than that reported for some other proteins.[21,22] Substantial energy migration apparently occurs and may indicate that the polypeptide chain is tightly folded.

The increase in $\lambda_{max,p}$ in this pH region indicates that the tryptophane groups become increasingly exposed to solvent as the tyrosyl groups ionize. No gross changes in conformation occur in this pH region, as ultracentrifuge studies have shown that the sedimentation coefficient remains constant in this range.[7]

CD studies[4] show changes in the mean ellipticity of the tyrosyl and phenylalanyl bands but not the tryptophane bands in this pH range. The results of the fluorescence, CD, ultracentrifuge titration studies are consistent, indicating that only minor conformational changes within the

subunits occur between pH 9 and 10.5. The fluorescence results suggest a decompacting of the individual subunits, allowing more permeation by solvent and TNS. As this decompacting occurs concomitantly with titration of the tyrosyl groups, the tyrosyl groups may play an important role in the cross linking within the individual subunits. Linkages of the type, $RC_6H_5OH\cdots NR'$, where N is the ε amino group of lysine, have been postulated by Lauffer,[23] who has suggested that such linkages within the individual subunits may stabilize the disaggregated protein relative to the aggregated protein. Protonation of the amino groups would then break the intrasubunit linkage and both components would be free to engage in intersubunit bonding. If such linkages do exist, then deprotonation of the tyrosyl group would also break the linkages but need not lead to aggretion for a number of reasons, e.g., electrostatic repulsion between the negatively charged subunits, or the failure of the amino group to obtain a positive charge.[24] While one could postulate a replacement linkage of the type, $C_6H_5\ddot{\underset{..}{O}}:\cdots H-\overset{H}{\underset{..}{N}}-R$, this would require association between two regions of high electron density.

Above pH 10.5, major changes in conformation appear to take place. The value for $\lambda_{max,p}$ increases to 350 nm, the same λ_{max} for proteins denatured by 8 M urea or 6 M guanidine HCl. I_T also increases rapidly as pH increases, despite the increasing electrostatic repulsion between TNS and the protein. This increase in I_T is probably due to exposure of the very hydrophobic binding sites of the interior of the protein. For comparison, we have also denatured TMVP at pH 7 by immersing a sample in a 60° water bath for 1 min, at which point the solution became cloudy. Fluorescence was then measured when the solution cooled. $\lambda_{max,p}$ increased to 340 nm, I_T increased by a factor of twenty, while I_p decreased by 20%.

These findings offer further supporting evidence that large increases in $\lambda_{max,p}$ and I_T indicate substantial protein denaturation.

The steady decrease in I_p with increasing pH, ceases around pH 11 and I_p rises slightly and then declines again. A possible explanation for this behavior is that the extensive unfolding of the protein above pH 10.5 brings the tryptophane residues somewhat out of the quenching spheres of the tyrosylate groups. At even higher pH the tryptophane groups themselves may ionize and become non fluorescent.[25]

Measurements made of the CD[4] of TMVP have also indicated that denaturation occurs above pH 10.5 and ultracentrifuge studies[7] support this conclusion.

In the pH range 7-6.5, a sharp decrease in I_p and a large increase in I_T occur. Aggregation of the protein into large helical rods occurs in this region,[1] and the fluorescence changes appear to be associated with the aggregation. As no change in $\lambda_{max,p}$ accompanies these fluorescence changes it seems likely that no major conformation change within the individual subunits occurs in this region. While it might be thought that the increase in I_T reflects a conformational change, evidence will be given in the subsequent paper,[26] linking this increase in I_T to the charge change associated with the aggregation. The decrease in I_p will also be discussed in this subsequent paper. One hydrogen ion per subunit is taken up in this region.[10] CD measurements have shown changes in the mean residue ellipticities of the phenyl and tyrosyl groups in this region.[4]

In the pH region 6-3 there is no change in $\lambda_{max,p}$, indicating that no major change in conformation occurs in this region. Three hydrogen

ions are taken up in this range,[10] which could, because of coulombic attraction between TNS and TMVP, lead to the increase in I_T. There is, however, also a small decrease in I_p, in this region. Such decreases in I_p have been reported for other proteins, and it is difficult to assign to them a structural cause.[21]

Below pH 3, $\lambda_{max,p}$ rises sharply and I_p decreases more rapidly than in the previous pH range. Both facts indicate that acid denaturation is occurring and acid denaturation is known to occur in this region.[1] The large increase in I_T which occurs in this region probably reflects the exposure of the hydrophobic sites and the increased positive charge on the protein. A solution of TNS in 90% dioxane, of the same concentration as that used in the pH experiment, had a fluorescence intensity and λ_{max} nearly the same as that of TNS + TMVP at pH $\leqslant$ 2.5. TNS in 90% dioxane has a quantum yield of fluorescence of 0.3,[11] so that TNS + TMVP at pH 2.5 also has a quantum yield near 0.3. Almost all of the TNS must be bound at this pH for the solution to exhibit such a high quantum efficiency.

TMV. A similar study was carried out using TMV instead of TMVP. Control experiments showed that TNS does not bind to RNA at the concentrations present in this experiment. The results of the experiments with TMV are plotted in Fig. 1. These results, when compared with those obtained for TMVP, show some significant differences. Perhaps most noteworthy is the lack of a sharp break in the curves of I_p and I_T in the pH range 7-6.5. As no changes in aggregation occur with TMV in this region, these data strengthen the conclusion reached above that the breaks in the corresponding curves of TMVP are associated with the aggregation of the protein.

While I_T for TMV also increases at acid pH, the increase occurs at somewhat lower pH than the corresponding increases for TMVP. It has been shown that at pH below the isoelectric point of TMV or TMVP, ca. 4.7,[10] TMV titrates at somewhat lower pH than TMVP. The increase in I_T at somewhat lower pH for TMV than TMVP implies then that electrostatic attraction between TNS and TMV in the acid pH range contributes actively to the binding.

In the pH range ≤ 3, no increase in λ_{max} of TMV occurs and I_T does not reach the maximum value which it does as when TNS binds to TMVP in the same pH range. It appears that TMV is more stable towards acid than TMVP. The differences between TMV and TMVP in their ability to bind TNS at pH ≤ 3 may then reflect the greater exposure of hydrophobic sites on TMVP.

There is a gradual decline in I_p with decreasing pH. This effect was also shown by TMVP and is discussed above.

In the alkaline pH range also, TMV is more stable than TMVP. The decrease in I_p which was related to the ionization of the tyrosyl groups begins at a slightly higher pH value. This fact is in agreement with data showing that the tyrosines in TMV titrate at higher pH values than they do in TMVP.[7] Also of interest is that I_T and $\lambda_{max,p}$ do not increase sharply until the pH of the solution is greater than 10.5. These results correspond well with the report that disassembly of the virus occurs around pH 10.5, and titration of the tyrosyl groups occurs when the protein subunits are released from the virus.[7] Further correspondence between the fluorescence data and the titration data is seen in the fact that the curves of I_p, I_T and $\lambda_{max,p}$ for TMV and TMVP are nearly

identical above pH 10.5, indicating that the same species is being titrated, i.e., the protein subunits. The titration curves for TMV and TMVP are also very similar after the protein subunits have been released from the virus.[7]

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Figure Caption

Fig. 1. Effect of pH on the intensity, I_p, and maximum wavelength, $\lambda_{max,p}$ of TMV and TMVP fluorescence and the effect of pH on the intensity of fluorescence of TNS, I_T, bound to TMV and TMVP. TMV and TMVP were excited at 280 nm and TNS was excited at 330 nm. TMV and TMVP solutions were 1 mg/ml and the concentration of TNS was 2.0×10^{-5} M.

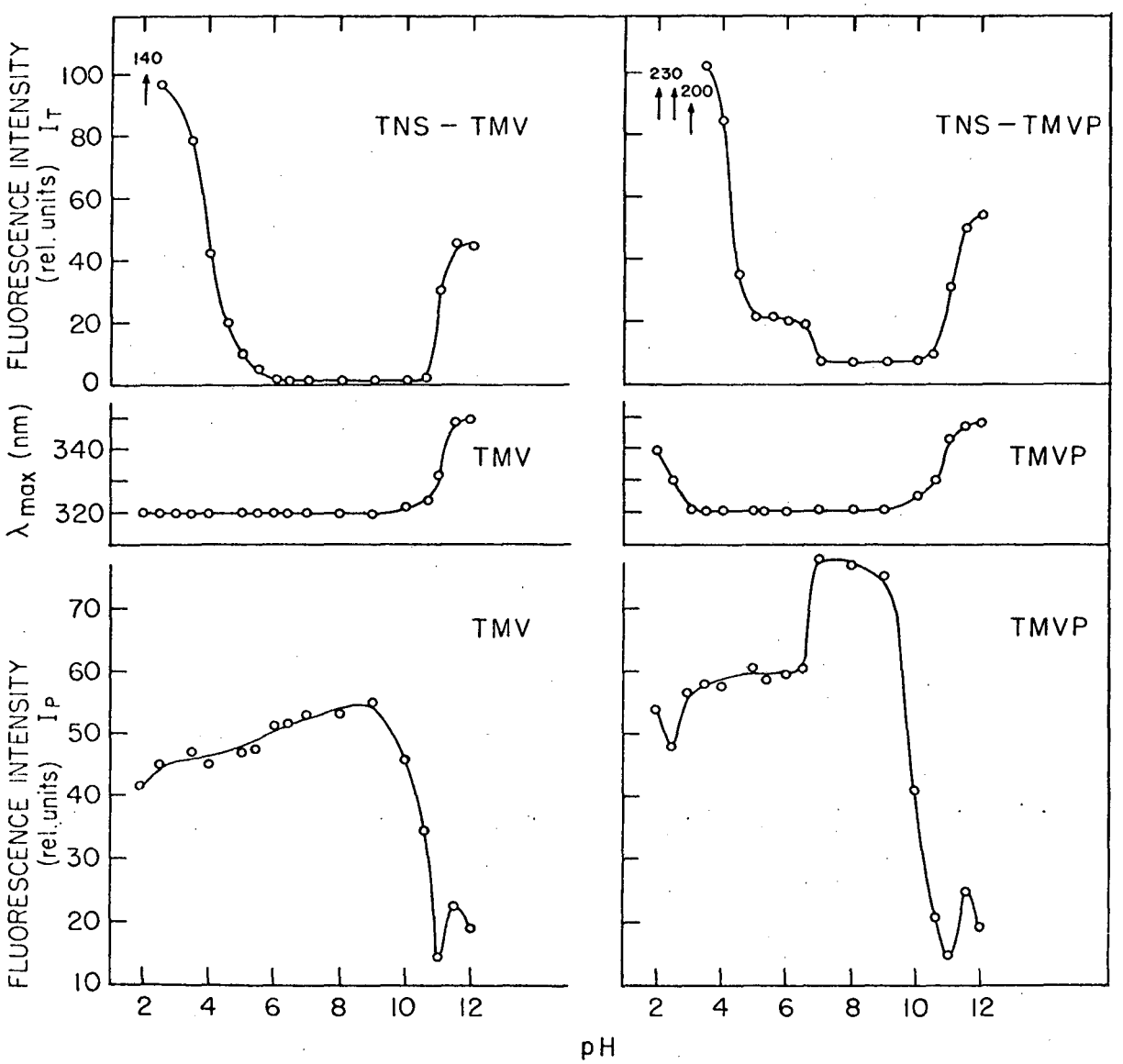

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Fig. 1

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