Carbon-13 Nuclear Magnetic Resonance Studies on Tobacco Mosaic Virus and Its Protein†

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ABSTRACT: Fourier transform nuclear magnetic resonance studies on 12% ^{13}C-enriched tobacco mosaic virus (TMV) and its rod-like protein oligomers in solution with molecular weights up to 42×10^6 are reported. In the virus ~17% of the carbons of the protein subunit have line widths of ≲300 Hz and $T_1 \lesssim 1$ s and are concluded to be mobile with more than one degree of freedom of internal rotation about a carbon–carbon bond. In the rodlike polymer of TMV protein at pH 5.3, 30% of the carbons are mobile, which implies rotational motions about carbon–carbon bonds and/or motions of the protein subunits within the polymer. The presence of internal mobility is supported by the observation that 20% of the carbons in the double disklike oligomer show decreasing line width upon increasing temperature; the remaining resonances have line widths which are temperature independent during the double disklike polymerization process. Since the molecular weight of TMV protein polymers increases with increasing temperature, this demonstrates that all nuclei within the double disklike oligomer are mobile. NMR and X-ray data on the double disklike polymer reveal differences with respect to internal mobility.

Tobacco mosaic virus (TMV) is a well-known plant virus (molecular weight 42×10^6) with a length and radius of 300 and 9 nm, respectively, containing 2200 identical protein subunits (molecular weight 17 500) complexed in a helix with 6600 RNA nucleotides (Finch, 1972). Both the virus and its coat protein oligomers (Durham, 1972) have been widely studied by various techniques such as electron microscopy (Durham & Finch, 1972), circular dichroism (Schubert & Krafczyk, 1969), sedimentation analysis (Paglini & Lauffer, 1968), and X-ray diffraction (Champness et al., 1976; Mandelkow et al., 1976; Stubbs et al., 1977).

Three-dimensional electron density maps for crystals of stacks of protein double disks and gels of the oriented virus with 0.5- and 0.4-nm resolution, respectively, have recently been obtained (Champness et al., 1976; Stubbs et al., 1977). Since the virus assembly occurs in solution, it is necessary to compare the molecular models obtained from X-ray studies of the virus and protein with those in solution. As has been previously shown, Fourier transform nuclear magnetic resonance (FT NMR) can be used for this purpose (Campbell, 1977; Robillard et al., 1976).

At first sight, NMR cannot be considered to be the appropriate method to study TMV and its protein in solution since it has usually been assumed that this technique can only be applied successfully to fairly small protein molecules (Oldfield et al., 1975a). For proteins with molecular weights ≳50 000, one expects long spin–lattice relaxation times (T_1) and short spin–spin relaxation times (T_2). This would result in a loss of signal-to-noise ratio (S/N) and spectral resolution.

This paper reports and discusses ^{13}C NMR results for isotopically enriched whole TMV particles and its coat protein in the form of double disks and helical rods.

Materials and Methods

TMV and Protein Preparation. TMV was purified according to Leberman (1966). A single sedimentation coefficient of 130 S was found; electron micrographs of the virus preparation reveal rod-shaped particles with a length of 300 nm, in full agreement with previous reports (Finch, 1972). TMV protein was prepared by following the method of Durham (1972) with slight modifications (de Wit et al., 1978). The $E_{280/252}$ ratio of the protein solutions in 0.12 M Tris-HCl, pH 8.6, is ≥2.5, and the solutions showed a single boundary (3.8 S) in the analytical ultracentrifuge and a single band with polyacrylamide gel electrophoresis at 5 °C under the same conditions. Protein concentrations were determined by using an $E_{280}^{1\%}$ of 1.27 (Durham, 1972). All samples were 12% ^{13}C-enriched by using $^{13}CO_2$ as the carbon source (J. L. de Wit, N. C. M. Alma-Zeestraten, and T. J. Schaafsma, unpublished experiments).

Analytical Ultracentrifuge Experiments. Analytical ultracentrifuge experiments were carried out by using a Spinco Model E ultracentrifuge equipped with Schlieren optics and automatic speed and variable temperature control. Sedimentation coefficient values were uncorrected for concentration, viscosity, and temperature.

Carbon-13 NMR Experiments. All ^{13}C NMR experiments were performed on a SPX Bruker supercon spectrometer in the FT mode by employing ~5 W continuous wave ^{1}H decoupling power with a 5-kHz bandwidth, an observed frequency for ^{13}C of 90.5 MHz, and quadrature detection. A D_2O lock proved to be unnecessary for our measurements, so D_2O isotope effects can be avoided by using H_2O solutions. The optimum ^{13}C enrichment at which no appreciable carbon–carbon spin–spin coupling is present is 10–15% (Wüthrich, 1976).

Results and Discussion

The 90.5-MHz ^{13}C Spectra of TMV. The ^{13}C NMR spectrum of 12% enriched TMV is given in Figure 1A. No significant saturation of the protonated ^{13}C resonances (120–200 ppm) is observed with a pulse repetition time T = 1 s. Under these conditions, there is partial saturation of nonprotonated carbon resonances (10–40 ppm). When T is increased to 2 s, the S/N ratio does not perceptibly increase with respect to its original value (4.5). This implies that $T_1 \leq 1$ s (Freeman & Hill, 1971); Figure 1B represents the ^{13}C NMR spectrum of a transparent solution of TMV dissociated

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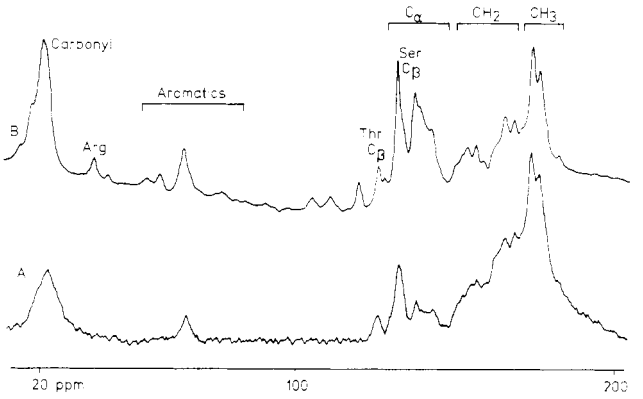

FIGURE 1: Broad-band ^{1}H-decoupled 90.5-MHz ^{13}C NMR spectra of both TMV and its RNA and protein constituents taken at ~30 °C with a concentration of 60 mg/mL (1-mL total volume) and 50 000 accumulations with a spectral repetition time of 1 s and a sensitivity enhancement of 30 Hz. The parts per million scale is referenced to CS_2, assuming 125.8 ppm for the β-Thr carbon position. The vertical scales of parts A and B are different. Further conditions: (A) 1 mM sodium phosphate, pH 7.2; (B) 1 mM sodium phosphate, pH 11.

into its RNA and native protein oligomers (molecular weight <50 000). The intensities of backbone (120–150 ppm) and aliphatic (150–200 ppm) carbon resonances in the spectrum of native TMV (Figure 1A) are 6 and 17%, respectively, of the corresponding resonances in Figure 1B, which represents the spectrum of a solution containing the free RNA and native protein oligomers. Intensities were calibrated by using a known amount of lysozyme (Oldfield et al., 1975a). As expected, the intensities of the RNA resonances (40–125 ppm) in Figure 1B are low due to secondary structure.

Apart from the large intensity drop, which is observed for all resonances in A vs. B of Figure 1, there is also a striking decrease in the intensity ratio of C_α and C_β resonances as follows from a comparison between the intensities in the 120–150- and 150–200-ppm regions in both cases.

For an interpretation of these results, we first consider the spin relaxation properties of rigid molecules.

The line width parameter $(1/\pi T_2)$ for ^{13}C–H dipole–dipole relaxation in large rigid proteins for $\omega_c^2\tau_R^2 \gg 1$ is given by eq 1 (Oldfield et al., 1975b), where γ_C and γ_H are the gyromagnetic ratios in radians per second for ^{13}C and ^{1}H, respectively, τ_R is the rotational correlation time of the protein, r_{CH} is the C–H distance, ω_C is the ^{13}C Larmor frequency in radians per second, N is the number of protons interacting with a ^{13}C nucleus, and $(\mu_0/4\pi)^2\gamma_C^2\gamma_H^2\hbar^2/r_{CH}^6 = 2.147 \times 10^{10}$ s^{-2} with $r_{CH} = 0.109$ nm. For spherical proteins, τ_R can be calculated from the Stokes–Einstein equation (eq 2) (Dwek, 1973), where η is the viscosity, M_r is the molecular weight, and $\bar{v}$ is the partial specific volume.

$$1/\pi T_2 = 0.2(\mu_0/4\pi)^2 N\gamma_C^2\gamma_H^2\hbar^2\tau_R/r_{CH}^6 \quad (1)$$

$$\tau_R = M_r\bar{v}\eta/(RT) \quad (2)$$

If the virus had a spherical shape, its ^{13}C resonances should be broadened by a factor 800, as compared to the resonance line width of native protein oligomers (molecular weight <50 000) and free RNA, assuming η and $\bar{v}$ to be the same in both cases.

For nonspherical TMV, τ_R(lateral) = 1.1×10^{-3} s (Tanford, 1961), yielding τ_R(axial) $\approx 1 \times 10^{-4}$ s. By use of these data and consideration of deviations from Stokes–Einstein behavior (Hallenga & Koenig, 1976), the broadening factor of 800 is a lower limit (Tao, 1969).

From dipole–dipole relaxation of a C–H fragment (Oldfield et al., 1975) T_1 is calculated to be >2000 s.

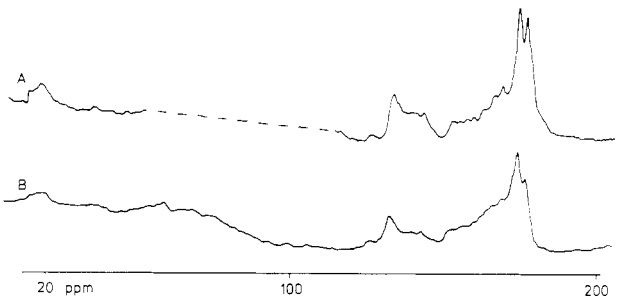

FIGURE 2: Broad-band ^{1}H-decoupled 90.5-MHz ^{13}C NMR spectra of TMV protein oligomers with a concentration of 60 mg/mL (1-mL total volume) in 0.1 M NaCl taken at 7 °C with a spectral repetition time of 0.5 s, a sensitivity enhancement of 45 Hz, and 17 000 as the number of accumulations. The parts per million scale is referenced to CS_2, assuming 125.8 ppm for the β-Thr carbon position. The vertical scales of parts A and B are different. Further conditions: (A) pH 10.0, the aromatic region is omitted because of a large hump in the spectrum; (B) pH 5.3.

These calculations demonstrate that under our NMR conditions (receiver dead time of ~50 μs and short repetition times) resonances of a rigid virus should all be broadened and saturated beyond detection (Torchia & VanderHart, 1976).

However, as is apparent from Figure 2A, ^{13}C resonance line widths are much smaller than those predicted for rigid TMV. From the 100-MHz ^{1}H NMR virus spectrum it is calculated that the observed aliphatic resonances in the ^{13}C NMR spectrum of the virus have line widths of <300 Hz (de Wit et al., 1978). Also, T_1 of the observed ^{13}C resonances is ≲1 s, in contrast with the value predicted above.

Apparently, the observed resonances cannot originate from a rigid virus particle. The experimental values of $(\pi T_2)^{-1}$ and T_1 yield a τ_R value which is much too short to be reconciled with the virus molecular weight. The observed relatively small values of $(\pi T_2)^{-1}$ and T_1 suggest that they arise from internally mobile regions in the protein subunits.

In view of the features of the ^{13}C NMR spectrum (Figure 1A,B), the observed species must be a ^{13}C-enriched protein; a single sedimentation coefficient (130 S) is found, implying that this species must be associated with TMV or that it is TMV itself.

Furthermore, it can only be a protein generated by fast biosynthesis in view of our experimental conditions for enrichment (J. L. de Wit, N. C. M. Alma-Zeestraten, and T. J. Schaafsma, unpublished experiments).

For different virus preparations following standard procedures (Leberman, 1966), the relative amount of the species observed by ^{13}C NMR is a constant and sizable (~10%) fraction of the total amount of TMV, which is known to be almost exclusively formed in infected plant cells. The method of preparation of TMV samples might also result in virus particles with a modified structure. X-ray maps of TMV samples prepared in an identical manner as samples used for NMR experiments do not show any sign of the presence of such particles (Stubbs et al., 1977). Of course, this argument is based on the plausible assumption that there is a close connection between structure and internal mobility. Finally, our TMV samples were insensitive for RNase treatment.

These arguments identify the observed resonances as those of TMV protein, as opposed to impurities and/or virus particles with a modified structure.

pH-Induced Rodlike Protein Polymerization. Parts A and B of Figure 2 represent the ^{13}C FT NMR spectra of small oligomers (3.8 S) and polymers of TMV protein, respectively. The protein polymer has a rodlike configuration with the protein arranged in a helix (Mandelkow et al., 1976). An

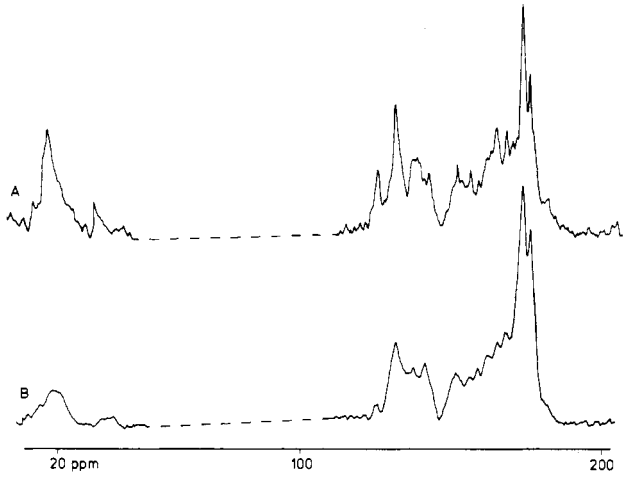

FIGURE 3: Broad-band ^{1}H-decoupled 90.5-MHz ^{13}C NMR spectra of TMV protein oligomers in 0.1 M NaCl at pH 7.3. The parts per million scale is referenced to CS_2, assuming 125.8 ppm for the β-Thr carbon resonance position. The aromatic regions are omitted because of large humps in the spectra. The vertical scales of parts of A and B are different. Further conditions: (A) 30 °C, 40 mg/mL, 4000 accumulations with $T = 1$ s, and a sensitivity enhancement of 40 Hz; (B) 7 °C, 60 mg/mL, 17 000 accumulations with $T = 0.5$ s, and a sensitivity enhancement of 45 Hz.

average intensity decrease of 70% is found for Figure 2B with respect to Figure 2A. This intensity drop cannot be accounted for by the increase of line widths, as is even apparent from superficial inspection.

In analytical ultracentrifuge experiments no components with sedimentation coefficients of <40 S have been found under the conditions given in the caption of Figure 2B, in agreement with literature data (Lauffer, 1975).

From these sedimentation coefficients, an increase of molecular weight of at least 40 is calculated when going from 3.8 to >40 S polymers. T_1 and T_2 should increase and decrease with this factor, respectively, if the coat protein oligomer is considered to be rigid. This again should result in saturation and severe broadening of the ^{13}C resonances. Therefore, the residual intensity of about 30% must also arise from mobility within the protein polymers (de Wit & Schaafsma, 1978).

Temperature-Induced Double Disk-Type Protein Polymerization. Under "physiological" conditions, polymerization of TMV protein results in a double disklike configuration, which is an important intermediate in the virus assembly process (Lauffer, 1975). In parts A and B of Figure 3, ^{13}C NMR spectra of oligomers with sedimenation coefficients of 18 and 4 S are shown. A number of ^{13}C resonances (corresponding to ~20% of the total integrated intensity) exhibit narrowing upon an increase of temperature, as is evident from a comparison of parts A (30 °C) and B (7° C) of Figure 3. For the remaining ^{13}C aliphatic resonances we conclude from ^{1}H NMR spectra at 100 MHz that no disappearance of spectral intensity occurs upon double disk-type polymerization; line broadening is not observed either, but our experiments do not allow us to decide between a constant line width or line narrowing due to severe resonance overlap (de Wit et al., 1978). From the similarity of the spectral envelopes in parts A and B of Figure 3, we may extend our previous conclusion to the C_α region as well.

Similarly as for the virus resonances, we want to exclude denatured protein subunits as the cause of the sharp resonances—constituting 20% of the total intensity—observed in Figure 3. Since the polymerization behavior of the sample exactly copies that of TMV protein (Lauffer, 1975), the observed molecules must all be functionally identical with TMV protein, implying that the observed resonances can arise neither from an impurity nor from a denatured fraction of the protein subunits.

Therefore, we may conclude that—similarly to the situation in the virus and the rodlike polymer—there must be also mobile regions in the protein double disks.

Internal Mobility. For a rather simple description of the effect of internal mobility on T_1 and T_2, we adopt Doddrell's model (Doddrell et al., 1972) as a starting point. This model is based on a correlation time for one degree of freedom (τ_g) for rotation of a tetrahedral C–H fragment about its C–C axis over 360° and τ_R for isotropic reorientation of the molecule. Strikingly, the model predicts that at 90.5 MHz T_1 becomes almost independent of τ_R and, simultaneously, T_2 linearly depends on τ_R if $\tau_R > 10^{-7}$ s and $\tau_g < 10^{-9}$ s. Then, T_2 is given by eq 1 with a reduction factor of 1/9.

According to van Putte (1970) a similar dependence of T_1 on τ_g is found when τ_g describes restricted rotations over angles much less than 360°, which may occur in native proteins (McCammon et al., 1976).

Rotational Degrees of Freedom within TMV and Its Protein Oligomers. The experimental value of T_1 (~1 s) for the virus can be accounted for by small angle rotations about C–C bonds (Doddrell et al., 1972; van Putte, 1970; de Wit et al., 1978). From the ^{13}C line width of 300 Hz for virus carbons we calculate τ_R to be 1.1×10^{-6} s, using Doddrell's model (1972) and assuming $\tau_g < 10^{-9}$ s. This τ_R value is still ~100 times smaller than the smallest of both hydrodynamic rotational correlation times (1×10^{-4} s), indicating that in the protein subunit regions, which exhibit internal mobility and thus are observable by NMR, rotation of C–H vectors occurs about more than one C–C bond.

An impression of which type of carbons is involved in these rotational motions can be obtained by comparing the weighted areas of different regions in the spectra of parts A and B of Figure 1. The observed 17% of the amino acid side chains (148–200 ppm) correspond to ~50 aliphatic carbons: ~50 CH_3 carbons between 172 and 200 ppm and ~30 CH_2 and CH carbons between 148 and 172 ppm. Aproximately 10 α carbons are observed in the region 120–150 ppm, comparable to the number of observed carbonyl resonances. The accuracy of these calculations is estimated to be within ±20%. The largest error arises from the determination of the relative spectral intensities in parts A and B of Figure 1.

Backbone carbon rotations with more than one degree of freedom also force connected carbons into rotational motion. Therefore, α carbons observed in the spectrum of the virus must belong to mobile regions of the protein subunits.

Upon polymerization of TMV protein into rods, a reduction of intensity for the ^{13}C NMR spectra is observed with only minor changes in spectral position and line width. This may be due to the absence of RNA in TMV protein which allows other modes of internal motion. Although spectra of the rodlike protein polymers at pH <5 still have ~10% of the original spectral intensity, their spectral shape then is considerably different.

We ascribe the line width narrowing of 20% of the double disk resonances (pH 7.3) upon an increase of temperature to temperature-induced motion of both backbone and side chains. This explanation is based on the observation that such a temperature increase is known to cause an increase of molecular weight (Durham, 1972), which would result in line broadening if the protein were rigid.

The fact that the remaining 80% of the carbons have a temperature-insensitive line width implies that their width is

not affected by molecular weight. This can only be so if these carbons participate in a motion which is decoupled from that of the entire polymer. Clearly, this requires more degrees of freedom of internal motion than contained in Doddrell's model. Such additional degrees of freedom for various types of RNA-free polymers (i.e., rods and disks) may involve motion within the protein subunit or motion of the protein subunit within the polymers (de Wit et al., 1978).

Conclusions

Apart from the conclusions presented in the previous discussion, there are some noteworthy implications for X-ray diffraction. The 0.4-nm resolution electron density map for the virus (Stubbs et al., 1977) is not inconsistent with internal mobility of amino acids, primarily on the exterior of the virus. Caspar & Holmes (1969) have reported small coordinated motions in the protein subunits of *Dahlemense* strain TMV, but to a much less extent and cut-off frequency than is indicated by the present NMR study. One expects similar results for the rodlike polymers, but only low-resolution X-ray data have been published (Mandelkow et al., 1976). The observation of a 0.5-nm resolution electron density map for crystals of stacks of double disks (Champness et al., 1976) at first sight contradicts our NMR data of double disks in solution, indicating that all nuclei within the disk are mobile; even if these nuclei possess only one degree of freedom of internal rotation, the electron density map of the protein subunit should be completely smeared out.

Note, however, that X-ray data for crystals of double disks have been obtained from 0.8 ionic strength solutions while NMR experiments were performed on oligomers in 0.1–0.2 ionic strength solutions containing double disks. It is conceivable that the protein in the crystal is "frozen" by the constraints of crystal packing and periodicity. Preliminary NMR experiments have shown that at high ionic strength the spectral intensity starts to disappear for the double disklike oligomer in roughly the same way as observed for the rodlike oligomer.

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