A ^{13}C and ^{1}H NMR STUDY ON ROD-LIKE POLYMERIZATION OF TOBACCO MOSAIC VIRUS PROTEIN

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1. Introduction

NMR studies in biochemistry have mainly been restricted to proteins with mol. wt $\lesssim 50\,000$ [1]. Due to limited resolution, the amount of structural information which can be obtained from such studies, even at 360 mHz, is rather restricted [2].

NMR can also be used for the study of molecular dynamical properties of biological systems with mol. wt $> 10^6$. The observation of spectral features itself, for example of tobacco mosaic virus (TMV) and TMV-protein [3,4], then contains information about the dynamical behaviour of nuclei within a protein subunit in such a biological system expressed in translational and rotational degrees of freedom.

Even in TMV, with a mol. wt 42×10^6, small numbers of ^{13}C nuclei of different types of amino acids are detectable with conventional Fourier Transform ^{13}C at 90.5 MHz because of their high degree of mobility [3]. For large biological systems ^{1}H NMR is inferior to ^{13}C NMR because of the much lower spectral resolution and the presence of a residual HOD resonance, even in 99.99% D_2O solutions. The sensitivity of natural abundance ^{13}C NMR is much lower than that for ^{1}H NMR, however. Therefore we have used 12% enriched TMV [3,5] in order to obtain ^{13}C spectra with sufficient signal-to-noise ratio.

This report is an extension of our earlier work, presenting the pH- and temperature-dependence of the rod-like polymerization in more detail.

2. Materials and methods

2.1. *Tobacco mosaic virus*

TMV was purified according to [6]. TMV-protein was prepared following [7] with slight modifications [4]. All protein solutions used in our experiments had an $E_{280}/_{252}$ ratio > 2.5 in 0.12 M Tris–HCl, pH 8.6, and showed a single boundary (3.8 S) in the analytical ultracentrifuge and a single band with polyacrylamide gel electrophoresis.

Analytical ultracentrifuge experiments were carried out using a Spinco model E ultracentrifuge equipped with Schlierenoptics, automatic speed and variable temperature control. *S* values are uncorrected for concentration, viscosity and temperature and have been determined with sedimentation velocity experiments at 22 000 or 40 000 rev./min.

TMV (strain Vulgare) was enriched up to 12%, using $^{13}CO_2$ as carbon source for photosynthesis in leaves of *Nicotiana tabacum* L (Samsun NN), inoculated with TMV. The enrichment procedure will be described in [8]. Enrichment up to 12% represents an optimum at which no noticeable carbon–carbon coupling is present [9]. $^{13}CO_2$ (90% enriched) and D_2O (99.9% enriched), were purchased from Bio Rad, NaOD (99% enriched) and DCL (> 99% enriched) were obtained from Merck. In D_2O solutions the normal pH meter readings have been taken [9].

2.2. *Nuclear magnetic resonance*

For ^{13}C measurements a Bruker SPX 360 supercon

spectrometer in FT mode with quadrature detection was used, employing ~ 5 W continuous wave ^1H decoupling power with ~ 5 kHz bandwidth, an observe frequency of 90.5 MHz, and 10 mm sample tubes. A ^1H lock turned out to be unnecessary for our experiments. ^1H measurements were carried out on a Varian XL 100 NMR spectrometer in FT mode with an external ^{19}F lock, an observe frequency of 100 MHz and 5 mm tubes. On both NMR systems constant temperature was maintained with a modified temperature control system allowing long time averaging experiments [4]. The receiver deadtimes of the both spectrometers was ~ 50 μs.

3. Results

3.1. *pH-induced rod-like polymerization*

The sedimentation coefficient of the protein polymers and oligomers at 7°C, pH 5.3 and pH 10.0 are > 40 S and 2.9 S, respectively. In the sedimentation velocity experiments at pH 5.3 a distribution of polymers with sedimentation coefficients 40 S, 70 S, 80 S and 90 S is found at 22 000 rev./min. Sedimentation coefficients < 40 S are absent at 40 000 rev./min. At pH 6.3 and 7°C, oligomers with sedimentation coefficients 15–18 S are present; below this pH and above this temperature oligomers with $S < 15$ have not been observed.

Figure 1 represents a ^{13}C spectrum of TMV-protein at 7°C and pH 10.0 in 0.1 M NaCl. The change in relative spectral intensities (measured by weighing the carbonyl, α-C and aliphatic regions) as a function of pH is shown in fig.2. The spectral regions of which intensities have been compared are indicated. Within measuring error this change is identical for the different spectral regions.

Figure 3 represents the drop in intensity for the aliphatic region of TMV protein in D_2O solution, upon rod-like protein polymerization as detected with ^1H NMR. Although in D_2O the polymerization behaviour of TMV-protein is considerably different from that in H_2O [10] the rod-like polymerization may also be expected to be a linear condensation polymerization process. As shown [4] the ^1H line shape at 100 MHz is much more sensitive to line broadening effects than the ^{13}C line-shape. Upon rod-like polymerization again no broadening of ^1H

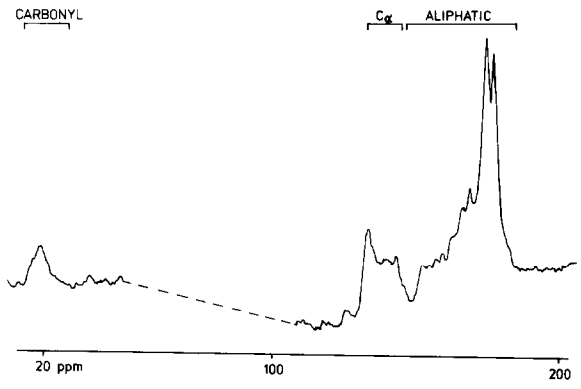

Fig.1. Broadband ^1H decoupled 90.5 MHz ^{13}C NMR spectrum of TMV protein taken at 7°C with conc. 30 mg/ml (1 ml total vol.) and 17 000 accumulations: acquisition time, 0.5 s; sensitivity enhancement, 30 Hz. The ppm scale is referenced to CS_2 assuming 125.8 ppm for the β-Thr carbon position. Further conditions: 0.1 M NaCl, pH 10.00. The aromatic region is omitted because of an interfering instrumental effect.

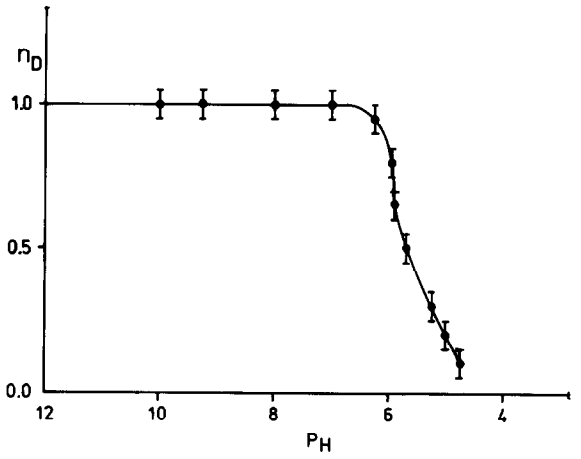

Fig.2. The ^{13}C intensity of TMV-protein for different spectral regions as a function of pH. The alpha-numerically labeled regions are indicated with arrows in fig.1. Conditions: 7°C, 0.1 M NaCl, 15 mM sodium phosphate and a protein concentration of 30 mg/ml. The NMR parameters are identical to those given in the caption of fig.1. n_D is the detectable NMR intensity. The error in n_D arises from the presence of spectral noise.

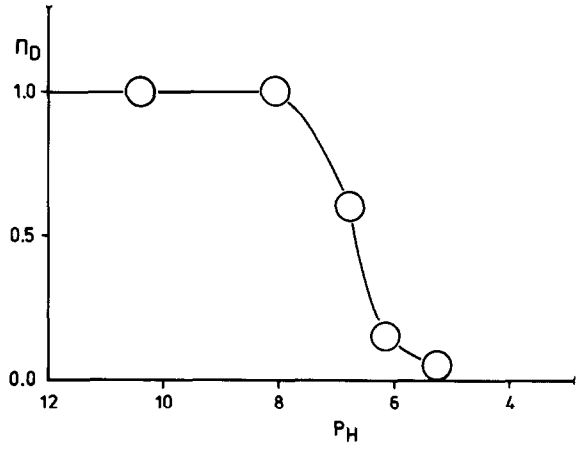

Fig.3. The ^1H spectral intensity of TMV-protein of the aliphatic region as a function of pD. Conditions are: 5°C, 0.2 M NaCl and a concentration of ~ 40 mg/ml in D_2O. The NMR parameters are: spectral width, 5000 Hz; accumulations, 10 000; sensitivity enhancement, 0.05 Hz; acquisition time, 0.5 s. Each graphical point represent the intensity of the largest group of resonances in the aliphatic region. n_D is the detectable NMR intensity. Experimental errors arise from the use of noise levels in the calculation of spectral intensities and from the fact that the pH values were measured outside the 5 mm NMR tube.

line shapes is observed within experimental error. The absence of line broadening in the ^1H and ^{13}C spectra cannot result from the receiver dead time of the NMR spectrometers, because the decrease in spectral intensity is identical for all spectral regions, independent of the line width close to the base line of a group of overlapping resonances. Absence of polymerization-induced line broadening therefore, cannot be due to a disappearance of spectral intensity through gradual line broadening beyond the limits set by the spectrometer.

The spectral intensity decrease upon lowering pH from 10–5.9 cannot be explained by a combination of limited acquisition time and increasing T_1 value, since spectra recorded with acquisition times 0.5 s and 1 s exhibit identical decrease of intensity with decreasing pH for the aliphatic region. Moreover, the intensity drop for non-protonated carbonyl carbons in ^{13}C NMR is comparable to that of the protonated carbons in the aliphatic region, despite the fact that the former have much longer T_1 values than the acquisition times emplyed in these experiments [4].

Figures 2, 3 and the analytical ultracentrifuge data therefore provide evidence that the pH-dependent intensity of the observable part of the ^1H and ^{13}C spectra is due to those nuclei within the rod-like polymers which are mobile enough to eliminate important line broadening [3,4].

3.2. *Temperature-induced rod-like polymerization*

At pH values close to 6 rod-like polymerization can also be induced by increasing temperature [12]. The ^{13}C spectral intensity change observed in the presence of such a polymerization process at different pH values and temperatures is presented in table 1 for carbonyl carbons only; identical results are found for the α-C and aliphatic regions. Since at high temperature all sedimentation coefficients (table 1) are > 30 S (corresponding to mol. wt $> 10^6$ [15]) spectra should be rigorously broadened [3] in the absence of internal motion.

A comparison of the effect of temperature on the spectral intensity (table 1) of large molecular weight polymers (> 30 S) in the range 20–30°C below pH 6.3 with the temperature effect on titration curves obtained [15] demonstrates that the absence of proton uptake observed by these authors upon increasing temperature in this range, is also reflected by the constant ^{13}C spectral intensity. The behaviour at pH 7.2 originates from polymerization to double disks and has been discussed [4]. Similar results are obtained for ^1H NMR of TMV-protein in D_2O solutions. Both for ^{13}C and ^1H spectra no line broadening upon temperature-induced polymerization is observed. Thus, the observed resonances again must arise from internally mobile polymers, such that no important line broadening is observed upon increasing molecular weight.

4. Discussion

Similar to what has been found for double disk-like polymerization [4], the exchange rate τ_m^{-1} of protein subunits between rod-like polymers is too small to account for the molecular-weight-independent NMR spectra [3]. For the present case, the Gibbs free energy of protein subunit binding within an polymer is $\Delta G = -76$ kJ [13], resulting in $\tau_m^{-1} < .35\ s^{-1}$

Table 1
^{13}C spectral intensity for the carbonyl region[a] at different values for pH and temperature[b]

pH	7.2	6.3	6.1	6.0	5.9	5.6	5.3
0°C		1					
7°C	1		1	0.8	0.6	0.5	0.3[c]
15°C	1	1	0.8				
20°C	1	0.7[c]		0.5[c]	0.3[c]		
25°C	1	0.7[c]	0.6[c]				
30°C	1	0.7[c]			0.3[c]	0.3[c]	

[a] The carbonyl region is shown in fig.1. Other conditions: conc. 30 mg/ml, 15 mM phosphate, 0.1 M NaCl

[b] The accuracy of the measured spectral intensity is ± 0.05 relative units as determined from the spectral noise level

[c] Only components present with sedimentation coefficients > 30 S

[4,14], where τ_m is the residence time of a protein subunit within the oligomer.

We may assume that the secondary and tertiary structure of the protein subunits within the polymer in solution must be largely maintained. Therefore, it has been suggested [3,4] that within protein oligomers motional degrees of freedom of amino acid side chains and backbone (within each protein subunit) and of protein subunits (within each protein oligomer) must be considered.

The pH- and temperature-dependent disappearance of spectral intensity (fig.1) up to pH 5.3, only differs within experimental error from the high pH spectrum by a scaling factor. Therefore, we propose that the main cause for the disappearance of spectral intensity upon pH-induced rod-like polymerization must reside in the loss of motional degrees of freedom of all nuclei in a protein subunit within the rod-like polymer. In this respect, our results do not allow to distinguish between motion within or of the protein subunits. Since no line broadening is observed upon lowering the pH, this loss of mobility must be abrupt, so that all carbon resonances of a protein subunit are either observed or have been broadened beyong detection. Similar to what has been found for double disk-like polymerization [4], the absence of line broadening upon rod-like polymerization in the pH range 5.3–6.3 at temperature > 20°C shows that the dynamics of the observable nuclei is independent of polymer molecular weight. Figure 2 and table 1 indicate that the change in spectral intensity is pH-controlled.

A two state model can describe this behaviour where in state N the nuclei in the protein subunits are 'locked' and non-detectable and in state D the nuclei in the subunits have suffient motional degrees of freedom to be detectable. The number of protein subunits in state D (n_D) depends on pH and is in equilibrium with the number of protein subunits in state N (n_N) according to the equation:

$$n_D + xH^+ \rightleftharpoons n_N \qquad (1)$$

where x is the number of protons which must bind to induce a subunit transition from state D to N.

By plotting log $[n_D/(1-n_D)]$ against pH, the pK and x of eq. (1) can be determined. From the resulting straight line, $x = 1.25 \pm 0.05$ and pK 7.0 ± 1 can be derived. Note that because $x = 1.25$ this pK does not simply represent the pK of the titrable groups within the protein subunits.

In agreement with [16,17] an uptake of ~ 3 protons/subunit has been shown [15], in particular by two titratable groups with raised pK values between 5.6 and 7.1 when TMV protein is titrated from pH 8.5–5 over a wide temperature range and 0.1 ionic strengh. An uptake of ~ 1.3 protons/subunit is observed when temperature is increased from 5–20°C [15] at pH 6.3, 0.1 ionic strengh. No further proton uptake is observed upon temperature increase to 30°C. Using a pK ~ 6.6 [15] the fractions of protein subunits with 0, 1 and 2 protons added can be calculated to be .10, .26 and .64 resulting in an

average uptake of 1.5 protons/subunit. Table 1 shows that the observable spectral intensity of 0.7 remains constant when temperature is further increased from 20–30°C. In view of the foregoing experimental results, the intensity of 0.7 shows that the addition of 1 proton has no effect on NMR intensity, in agreement with the calculated value of $x > 1$; the $n_D \rightarrow n_N$ transition, therefore, cannot be induced by the addition of the first of the ~ 3 protons which is taken up when titrating from pH 8.5–5.0.

The pK values of the titratable groups cannot be deduced directly from our NMR results although it is clear that they must be between 5.6 and 7.0.

The two state model presented above, does not give a complete description of the titration behaviour of titratable nuclei in TMV subunits. At pH 4.8, still ~ 10% of the ^{13}C nuclei is observable which is about 10-times more than predicted from the model presented above. This remaining intensity may well orginate from internal mobility, similar to that found for TMV [3]. In both cases the α-C resonances have strongly diminished intensity.

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