AN EPR STUDY OF THE KINETICS OF ENCAPSIDATION OF SPIN-LABELED POLYADENYLIC ACID BY TMV PROTEIN

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1. Introduction

The interaction of nucleic acids with virus proteins is a crucial step in the assembly of viruses. The interaction has been examined with a wide range of biochemical and physical techniques [1–7]. Among these techniques, the EPR spin-label method applied to the nucleic acids seems to be very promising, because this method is able to selectively monitor the nucleic acid. The most common problem is a disturbance of the experiment by protein–protein interactions.

It is known that TMV protein can reassemble with synthetic polynucleotides resulting in virus-like particles [8]. The rate of this reaction is ~2 orders of magnitude slower than with native TMV RNA [6]. Spin-labeled polyadenylic acid (SL-poly(A)) can be encapsidated by viral proteins resulting in short nucleo-protein particles [7].

We have used this system to study the kinetics of encapsidation of TMV protein and SL-poly(A) by recording the EPR spectrum of the reaction mixture at different time intervals. Also we have measured the time course of the turbidity of the sample.

From these experiments we are able to get information about the kinetics of the assembly reaction. We find that SL-poly(A) favorably reacts with A-protein in contrast to TMV RNA, in which disk-protein plays a significant role. We believe that this method is a valuable contribution to the range of techniques involved in the study of protein–nucleic acid interactions.

2. Materials and methods

2.1. *TMV protein*

The protein was isolated from TMV, strain vulgare as in [10] and dialyzed against a large volume of 0.1 M NaH_2PO_4 buffer (pH 7.0). The protein solutions were concentrated in an Amicon cell. All steps were performed at 4°C and fresh preparations were used for every experiment.

2.2. *Preparation of spin-labeled poly(A)*

Poly(A) (Sigma, 300–500 nucleotides long, as determined by gel filtration) was labeled with 3-(2-iodoacetamido)-2,2,5,5-tetramethyl-1-pyrrolidinyl-oxyl (Syva, SL 120) as follows: 4 mg SL 120, dissolved in 0.2 ml ethanol was mixed with 50 mg poly(A) in 1.8 ml, 0.05 M NaH_2PO_4 buffer (pH 6.6). The mixture was incubated for 5 days at 38°C in the dark (to prevent hydrolysis of the spin label) and was dialyzed against large volumes of 0.1 M NaH_2PO_4 buffer (pH 7.0) until no free spin label could be detected. With this procedure, 1 of every 260 nucleotides was labeled as determined in [9]. The EPR spectrum of the SL-poly(A) was essentially the same as that in [9].

2.3. *Assembly reaction*

We have used two different protein preparations:

(1) TMV protein incubated at 4°C, which we will refer to as 'overshoot' protein [11];
(2) TMV protein, incubated at room-temperature for ≥24 h, which we will call 'equilibrium' protein because of the equilibrium between disks and A-protein [12] under these conditions.

Each preparation was mixed with SL-poly(A) in a weight ratio of 20:1 in 0.1 M NaH_2PO_4 buffer (pH 7.0). Immediately after mixing the reaction mix-

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ture was transferred into a flat cell (Varian E248 aqueous solution sample cell), which was placed in the T110 cylindrical cavity of a Varian E6 EPR spectrometer. The cavity was kept at the desired reaction temperature by an air stream of constant temperature. The spectrometer was connected with a MINC-V microcomputer. After optimizing the position of the cell, spectra were recorded and stored on disk. Scan-times were always as short as possible (8 min) in order to obtain a 'continuous' reaction profile.

A second portion of the reaction mixture was put into a 1 mm quartz cuvette and placed in a Shimadzu UV-200 double-beam spectrophotometer, equipped with a constant temperature cell holder, kept at constant temperature by a circulation waterbath. The turbidity of the solution was measured at 360 nm.

3. Results

3.1. *EPR measurements*

EPR spectra of a series from one experiment are shown in fig.1. The spectra consist of a sharp and broad component arising from mobile and immobile spin labels, respectively. Several alterations in the spectrum can be distinguished in the progress of the reaction:

(i) A decrease of the peak-heights of the sharp components can be seen. This indicates an immobilization of the spin label, since simultaneously the contribution of the immobile components increases.

(ii) The ratio of the peak-heights of the low-field line and the high-field line h_{+1}/h_{-1} of the mobile part of the spectrum decreases from ~2 to 1, due to increasing mobility of the non-encapsidated spin label. A plot of the peak-heights of the h_{+1} lines and the h_{+1}/h_{-1} ratio *vs* time is given in fig.2A. A semi-logarithmical plot of the peak-height of the h_{+1} lines (fig.2B) results in one straight line with slope 1.7×10^{-3} min^{-1} for the 'equilibrium' mixture and two straight lines with slopes 1.6×10^{-2} min^{-1} and 1.0×10^{-3} min^{-1} for the 'overshoot' mixture, indicating first-order reactions.

3.2. *Turbidity measurements*

The course of the turbidity during the reaction is reproduced in fig.2C. The 'overshoot' effect is clearly visible for the 'overshoot' mixture. This is in good agreement with [13]. Note that the increase of turbidity in the first hour of the assembly reaction of the 'overshoot' mixture is faster than for the protein alone, because poly(A) is encapsidated by TMV protein.

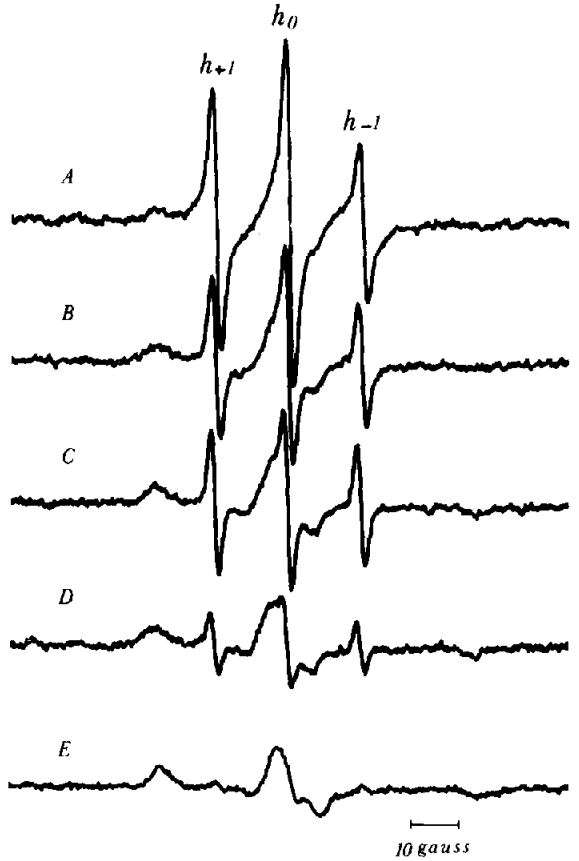

Fig.1. EPR spectra of a reaction mixture of 13 g TMV protein/l and SL-poly(A) in a weight ratio of 20:1 in 0.1 M NaH_2PO_4 buffer (pH 7.0), temperature raised from 4–25°C within 5 min. Times after start of reaction: (A) 15 min; (B) 100 min; (C) 260 min; (D) 460 min; (E) 24 h. The samples were contained in a flat quartz cell. EPR conditions: frequency, 9.40 GHz; microwave power, 10 mW; modulation amplitude, 1.0 G; scan time, 8 min.

4. Discussion

SL-poly(A) is encapsidated by TMV protein. This is manifested by the decrease of h_{+1} (fig.2A). The encapsidated SL-poly(A) is readily released by adding urea to 4 M final conc. to the reaction mixture. The resulting EPR spectrum is the same as the free SL-poly(A) spectrum (H. W. M. unpublished). The

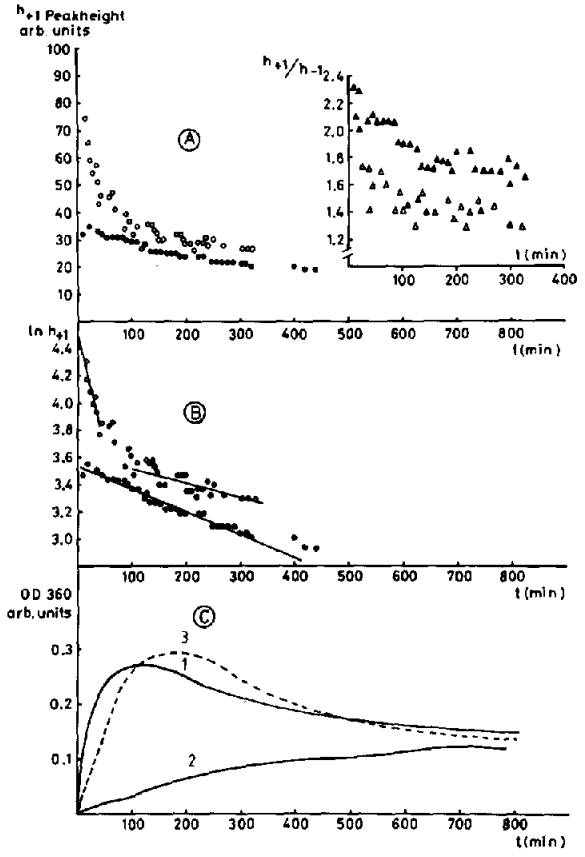

Fig.2. (A) Peak-heights of the h_{+1} lines of the EPR spectra of the 'overshoot' mixture (○) and the 'equilibrium' mixture (●) *vs* time. The inset shows the h_{+1}/h_{-1} ratio *vs* time of the 'overshoot' mixture (△) and the 'equilibrium' mixture (▲). (B) Semi-logarithmical plot of the data from (A). The straight lines are least-square fits to the experimental points. (C) Time course of the light scattering at 360 nm: (1) 'overshoot' mixture; (2) 'equilibrium' mixture; (3) 'overshoot' mixture without SL-poly(A).

peak-height of the h_{+1} line is a good monitor for the amount of non-encapsidated SL-poly(A) in the reaction mixture, since this line is almost isolated from the immobile component in the spectrum. For simplicity, we have assumed its linewidth to be constant during the reaction. Small effects of the changes in mobility of the spin label (fig.2A, inset) on the linewidth can be neglected. In this way the peak-height is proportional to the intensity of the line. Semi-logarithmical plotting of the peak-heights *vs* time results in straight lines (fig.2B), indicating first-order reaction kinetics.

The reaction in the 'overshoot' mixture shows two processes with different reaction rates. The fast process ($k_{fast} = 1.6 \times 10^{-2}$ min^{-1}) should involve A-protein, since it is known that under the preparation conditions (4°C, pH 7.0, 0.1 M ion strength) A-protein is the main (up to 95%) component [10]. Warming up the A-protein solution to 25°C at the start of the experiment results in the formation of large protein aggregates, as follows from the turbidity-plot (fig.2C). These large aggregates will eventually break down into disk-protein in hours [13]. At the maximum of the turbidity curve of the overshoot of A-protein the assembly reaction has slowed down (see fig.2B) and continues with a much slower rate. The rate of the slow ($k_{slow} = 1.0 \times 10^{-3}$ min^{-1}) reaction is about the same as the rate of the encapsidation by an 'equilibrium' mixture (fig.2B), consisting of >80% disk protein and 20% A-protein [10]. This slow reaction may therefore arise from the reaction of SL-poly(A) with disks. Another explanation is that the reaction still proceeds with A-protein that is released from disaggregating disks or larger protein aggregates, being now the rate-limiting step. The disaggregation of disks to A-protein upon dilution is a process of hours [14]. We might expect that the rate of disaggregation of disks, as a result of continuously removing the A-protein by the reaction with SL-poly(A) is about the same.

Another interesting observation is the increase of mobility of the non-encapsidated SL-poly(A) as monitored by the decrease of the h_{+1}/h_{-1} ratio (fig.2A, inset). A possible explanation for this is a destacking of the bases of the SL-poly(A) before the actual encapsidation takes place. This implies, however, that the growing of the nucleoprotein rod will start from a nucleus on the poly(A)-strand, causing a sort of stripping effect on the stacked bases.

It is clear that the encapsidation of SL-poly(A) by TMV protein must be a different process than the encapsidation of TMV RNA:

(1) The reaction of disk-protein with SL-poly(A) is ~100-times slower than the reaction with TMV RNA [6];
(2) This work shows that SL-poly(A) is favourably encapsidated by A-protein, >10-times faster than disk-protein, while the requirement of disks in the initiation of the assembly with TMV RNA is generally accepted [15].

These differences may be explained as follows: Since SL-poly(A) is much shorter than TMV RNA and only consists of adenosine residues, it possesses no specific

secondary structure, which fits the disk structure, as is the case for TMV RNA. In other words: poly(A) lacks the 'loop' necessary for a fast initiation process.

Our results strengthen the conclusion that the initiation of the assembly of TMV is a highly efficient process, due to the unique structure of the RNA as well as the spatial geometry of the protein aggregates.

From our experiments, it is clear that the EPR technique is a powerful tool in studying the kinetics of the encapsidation reaction of a polynucleotide and TMV protein, since it clearly distinguishes between effects of protein aggregation and nucleotide–protein interaction, in contrast to turbidity measurements.

References

[1] Favre, A., Guilley, H. and Hirth, L. (1972) FEBS Lett. 26, 15–19.
[2] Jacrot, B. (1980) Phil. Trans. R. Soc. Lond. B 290, 627–633.
[3] Lanina, T. P., Terganova, G. V., Ledneva, R. K. and Bogdanov, A. A. (1976) FEBS Lett. 67, 167–170.
[4] Lebeurier, G., Nicolaieff, A. and Richards, K. E. (1977) Proc. Natl. Acad. Sci. USA 74, 149–153.
[5] Stubbs, G., Warren, S. and Holmes, K. (1977) Nature 267, 216–221.
[6] Butler, P. J. G. (1972) J. Mol. Biol. 72, 25–35.
[7] Powell, A. T., Gordon, M. P., Caspary, W. J., Greene, J. J. and Ts'o, P. O. P. (1978) Nucleic Acid. Res. 5, 3977–3992.
[8] Fraenkel-Conrat, H. and Singer, B. (1964) Virology 23, 354–362.
[9] Bobst, A. M. (1972) Biopolymers 11, 1421–1433.
[10] Durham, A. C. H. (1972) J. Mol. Biol. 67, 289–305.
[11] Schuster, T. M., Scheele, R. B. and Khaizallah, L. H. (1979) J. Mol. Biol. 127, 461–485.
[12] Durham, A. C. H., Finch, J. T. and Klug, A. (1971) Nature New Biol. 229, 37–42.
[13] Vogel, D., Durham, A. C. H. and De Marcillac, G. D. (1977) Eur. J. Biochem. 79, 161–171.
[14] Butler, P. J. G. (1976) Phil. Trans. R. Soc. Lond. B 276, 151–163.
[15] Butler, P. J. G. and Durham, A. C. H. (1977) Adv. Protein Chem. 31, 188–251.