Bull. Inst. Chem. Res., Kyoto Univ., Vol. 69, No. 2, 1991.

Structural Kinetics: Temperature-Jump Synchrotron Small-Angle X-Ray Scattering Study of Tobacco Mosaic Virus Protein as an Example

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Received June 15, 1991

The measuring system for synchrotron small-angle X-ray scattering (SAXS) coupled with a temperature-jump (T-J) device was developed and applied to the study of the self-assembling process of tobacco mosaic virus protein(TMVP).

Firstly, the static SAXS measurements were performed to observe TMVP aggregates at several temperatures. The self-assembly process of TMVP was then investigated by rapid T-J time-resolved SAXS experiments. The T-J apparatus employed was designed so as to follow even the fastest assembling process of TMVP with a reasonable signal-to-noise ratio.

The quantitative formalism for TMVP rod formation was proposed, and the analysis of the experimental results was found consistent with the two-stage aggregation of TMVP with an equilibrium constant, and two rate constants. Proposed analytical procedure is applicable not only to the T-J method but also to the other kinetic (time-resolved) small-angle solution scattering experiments.

KEY WORDS: Small-angle X-ray scattering / Temperature jump device / Tobacco mosaic virus protein / Time-resolved experiments

INTRODUCTION

The small-angle X-ray solution scattering method is the best available to investigate structural changes of biological macromolecules in solution, for example, a reconstituting process of a virus from its constituents (nucleic acids and proteins), or a structural change in an enzyme accompanied by its enzymatic reaction. However, a laboratory-size (conventional type with an X-ray tube) small-angle X-ray scattering (SAXS) method requires more than several hours for measurements even using a position-sensitive proportional counter (PSPC) because of weak scattering intensities from solutions. Thus this method is not capable of observing a reaction process in a second-unit interval nor the structures of intermediate molecules formed during reaction, which would disappear in less than a few seconds, although the molecular

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structure will be evaluated with this method at the initial or the final state of reaction. An extremely strong X-ray beam generated by a synchrotron storage ring offers, when coupled with fast reaction technique, the possibility of kinetic observation of structural changes of biological macromolecules and their assemblies that play an important role in the appearance and control of biological functions. To observe such rapid structural changes, we have designed and constructed a SAXES (small-angle X-ray scattering equipment for solutions) system[1] fitted to the Beam Line-10C at the Photon Factory of the National Laboratory for High-Energy Physics in Tsukuba. The temperature-jump (T-J) apparatus has been constructed for installation in the SAXES system to perform the SAXS T-J experiments within a few hundred milliseconds[2].

Many studies have been undertaken to elucidate the association-dissociation reaction of tobacco mosaic virus coat protein (TMVP)[3-7]. Durham, Finch and Klug[8] have shown the phase diagram of the possible states of TMVP aggregation as a function of pH and ionic strength from the results of ultracentrifugal and electron microscopic observations. TMVP self-assembly states are also influenced by temperature: increasing temperature induces more aggregation states. In this work we have applied a time-resolved SAXES equipped with the T-J apparatus to investigate the assembly process of TMVP induced by temperature-jump. Static SAXS from TMVP solutions were observed in advance under different conditions in order to confirm the initial and final states of the TMVP aggregation process. The rapid assembly process was then investigated by the time-resolved SAXS measurements. The results were analyzed in terms of a set of kinetic equations which describe the two-step aggregation of TMVP with an equilibrium constant K_1 between A-protein and double-layer disks and two rate constants k_{+2} and k_{-2}, respectively, for association and dissociation of disks.

EXPERIMENTAL PROCEDURES

Materials

We have prepared TMVP from TMV, Japanese common strain OM[9] according to the method described by Hiragi *et al.*[10] The TMVP solutions for the SAXS measurements were dialyzed against phosphate buffers (5 to 100 mM sodium orthophosphate, pH 7.2), stored at 4°C and used within 1 week of preparation. Under this storage condition, no aggregate of more than 8S was observed by sedimentation velocity at 5°C.

Small-angle X-ray scattering

SAXS measurements were carried out with the optics and detector system of SAXES[1] installed at the 2.5 GeV storage ring in the Photon Factory. An outline of the system is illustrated in Fig. 1. The circulating current of electrons or positrons in the storage ring was 100 to 250 mA. A wavelength, λ, of 1.49 Å was used and the specimen-to-detector distance was about 1900 mm. The scattered X-ray was registered at 512 different angles from Q($Q=4\pi \sin\theta/\lambda$, where 2θ is the scattering angle) =

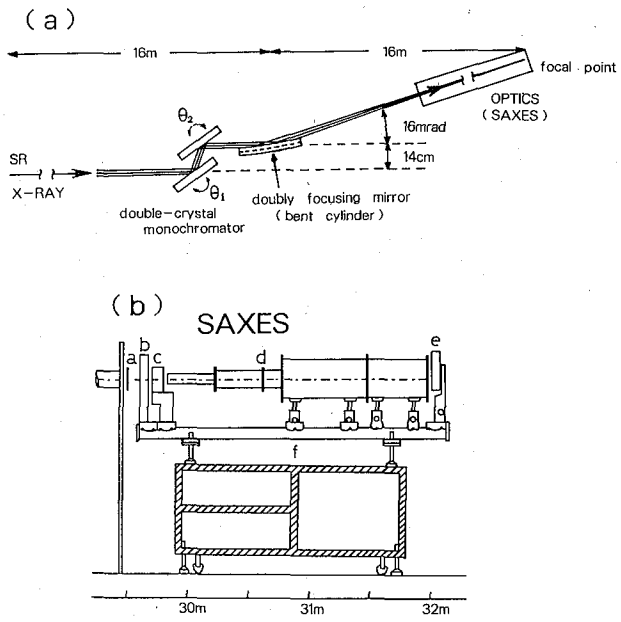

Fig. 1 (a) Optics of BL-10C consisting of a double crystal monochrometer and a bentcylindrical mirror. (b) Schematic drawing of the small-angle X-ray scattering equipment for solutions(SAXES). a; the exit window of X-ray beam, b; the third slit assembly, c; specimen holder, d; vacuum pipe, e; detector(PSPC), and f; double optical rails.

0.661×10^{-2} Å^{-1} to 0.334 Å^{-1} (the Bragg spacing equivalent to d_B=951 to 18.8 Å) by using the one-dimensional PSPC with an effective length of 160 mm (Rigaku Denki Co., Ltd.). The scattered intensities were corrected for the variation in the incident X-ray flux by monitoring the beam with an ionization chamber placed in front of the observing cell for measurements. The net intensities were obtained by subtracting the scattering intensities of a blank buffer solution from those of the protein solution. The focal size of the beam was small compared to the camera length, so that the smearing effect on the scattering profile was negligible as verified by simulation calculation. The CAMAC data acquisition system was controlled by a mini-computer MINC11/23 (Digital Equipment Co., Ltd.). Further analysis of the data was performed using a FACOM M380Q computer (Fujitsu Co., Ltd.).

Combination of a novel type of T-J device and the SAXES[2] enables the dynamic (time-resolved) measurement of scattered X-ray from the system undergoing the structural change within a few seconds. Fig. 2 depicts a schematic diagram of the combined system. The measurements were performed with the TMVP solutions of pH 7.2 in 50 mM and 100 mM phosphate buffer (corresponding approx. to ionic strength of 0.1 and 0.2 M, respectively), following the structural change of TMVP induced by the temperature-jump from 5.0°C to 20.0°C or to 25.0°C[11].

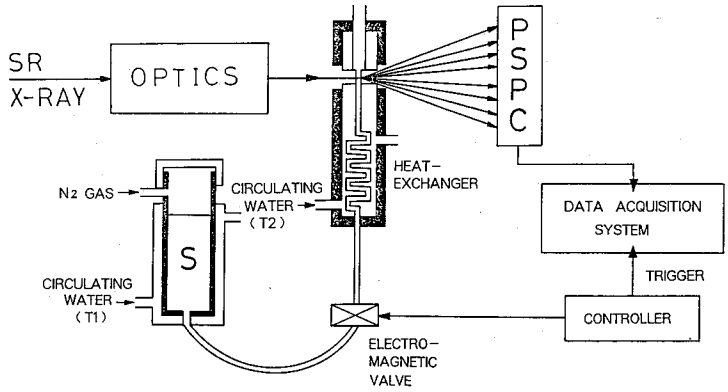

Fig. 2 A schematic diagram of the system for time-resolved temperature-jump synchrotron small-angle X-ray scattering measurement at the Photon Factory.

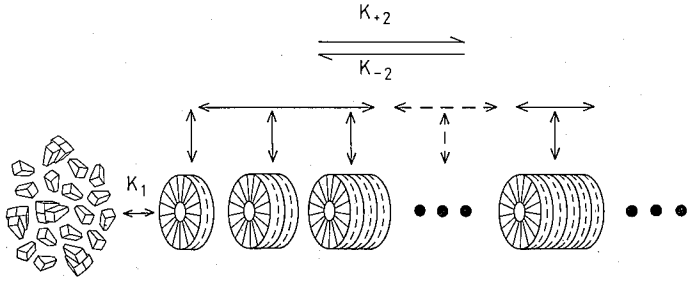

Fig. 3 Self-assembly scheme of TMV protein. The following assumptions are made for the self-assembling process: (1) 20S disks are formed instantaneously from A-proteins, where both components are in an equilibrium relation, and (2) further association takes place through random association-dissociation mechanism of stacking disks or rods with an association velocity constant k_{+2}, and a dissociation velocity constant k_{-2}.

Analysis of the experimental data

While the spectroscopic method determines the concentration of the initial, the intermediate or the final products of reaction directly, the small-angle scattering yields the various degree of the statistical averages of physical parameters of an ensemble in the course of the structural change. The conventional analysis employed for the spectroscopic method cannot be applied to the kinetic study of association-dissociation reaction of biological macromolecules observed by small-angle scattering. For example, the initial slope of the Guinier plots[12] yields the z-averaged radius of gyration $R_{g,z}$, and $J(0)/c$ is proportional to the weight average molecular weight M_w where c is the concentration of solute and $J(0)$ is the scattered intensity extrapolated to the zero angle. Thus, the analysis was made in terms of a set of kinetic equations that describe a two-step TMVP assembly process illustrated in Fig. 3. The first assembly step is considered as the formation of the 20S disks in equilibrium with A-protein and then the further disk stacking takes place as the association/dissociation process, that is:

(i) Equilibrium condition

Since formation of the 20S disks from A-protein (a small aggregate of TMVP subunit) is considered to be rapid, this process may be expressed in terms of an equilibrium constant K_1:

$$d_1/a^n=K_1 \quad (n=34/4) \tag{1}$$

Here d_1 and a denote the molarities of the 20S disk and A-protein, respectively, and the A-protein is assumed to be a tetramer[2] for simplicity. This assumption seems appropriate, since the mean number of polymerization for A-protein is a mixture of one to five subunits.

(ii) Dynamic differential equation

The disk stacking is specified by two rate constants for the association and dissociation of disks and stacked disks (short rods). A change in the concentration of the i-stacks of the 20S (double-layered) disk, $d_i(t)$ (see Fig. 3), is given by the differential equation:

$$\begin{aligned}\frac{\mathrm{d}(d_i(t))}{\mathrm{d}t} &= 2k_{+2}\sum_{1\leq j\leq i/2}^{m} d_j(t)\times d_{i-j}(t) \\ &\quad +2k_{-2}\sum_{j=i+1}^{m} d_j(t) \\ &\quad -\frac{(i-1)}{2}2k_{-2}\times d_i(t) \\ &\quad -2k_{+2}\times d_i(t)\sum_{i=1}^{m-i} d_j(t)\end{aligned} \tag{2}$$

where k_{+2} and k_{-2} denote the association and dissociation rate constants in the disk-stacking process (starting from the 20S disk), respectively, and m is the maximum number of stacking. The conservation of total concentration gives the constraint condition to solve the differential eq. (2).

(iii) Conservation of total concentration

The tetramer concentration $a(t)$ at the time t is determined by the conservation relation with an initial concentration of tetramer a_o:

$$a_o=a(t)+(34/4)\sum_{i=1}^{m} i\times d_i(t) \tag{3}$$

By use of the least-squares fitting program SALS[13], optimized values of K_1, k_{+2} and k_{-2} were determined by solving eq.(2) with the constraint of eq.(3) to yield the mean-square radius of gyration best fit to the observed value at each time-frame. Here the physical parameters such as the z-average mean-square radius of gyration and the weight average molecular weight are explicitly given in terms of the disks and A-protein as summarized below.

(iv) Mean-square radius of gyration

The value obtained from the Guinier plots corresponds to the z-averaged mean-square radius of gyration $R_{g,z}^2$(t) defined by:

$$R^2_{g,z}(t)=\frac{4^2\times a(t)\times R^2_{g,z}+34^2\Sigma d_i(t)\times R^2_{g,z}}{4^2\times a(t)+34^2\Sigma d_i(t)} \tag{4}$$

where $R_{g,A}$ is the radius of gyration of A-protein and $R_{g,i}$ of the i-stack of 20S disks.

(v) Weight-average molecular weight

$J(0)/c$ is proportional to the weight average molecular weight, M_w, given in terms of $a(t)$ and $d_i(t)$ as:

$$M_w(t)=\frac{4\times a(t)(4M_0)+34\Sigma d_i(t)\times(34iM_0)}{4\times a(t)+34\Sigma d_i(t)} \tag{5}$$

where M_0 denotes the monomer molecular weight.

Conventional evaluation of the integrated intensity, cross-sectional dimension and thickness of the aggregate was avoided in the present analysis in order not to introduce any ambiguity due to the polydispersity of the system. Although the analysis is model-dependent, the procedure may be applied to other systems undergoing the kinetic transition observed by X-ray scattering.

RESULTS

Static measurements

The static SAXS experiments were performed with the TMVP solutions (pH 7.2) at the protein concentration 5.0 or 12.0 mg ml^{-1} at 4.8, 10.2, 14.9. 19.8 and 25.0°C. Plotting logarithms of the excess scattered intensities against Q^2(Guinier plot[12]) revealed an almost linear line in the low Q^2 region, and yielded the z-average radius of gyration from a slope and the value $J(0)/c$ proportional to the weight average molecular weight from an intercept at $Q=0$. In general the values of $R_{g,z}$ increase with temperature, buffer strength and protein concentration (see Table 2 of reference 2). The trend of change in the z-average radii of gyration is roughly parallel to that of the sedimentation coefficients observed by sedimentation velocity measurements.

The TMVP aggregation was evaluated at various temperatures from the static SAXS measurements in order to specify the initial and the final stages of T-J experiments.

Dynamic measurements

Fig. 4 shows the fastest case of the assembly process in the present experiments. The Guinier plots of the solution (12.0 mg TMVP ml^{-1} in 100 mM phosphate buffer and a T-J from 5.0°C to 25.0°C) are depicted for each time-frame with the exposure time of 0.8 sec and show a signal-to-noise ratio good enough for the analysis with a total accumulated counting time of 16 sec for each frame. Here a series of 20 experiments were repeated and the scattered intensities were accumulated.

From a Guinier plot such as Fig. 4, we evaluated the z-averaged radius of gyration and the value proportional to the weight average molecular weight as a function of time. A time course of $R_{g,z}$ was obtained as shown in Fig. 5 by circle, where $R_{g,z}$ are

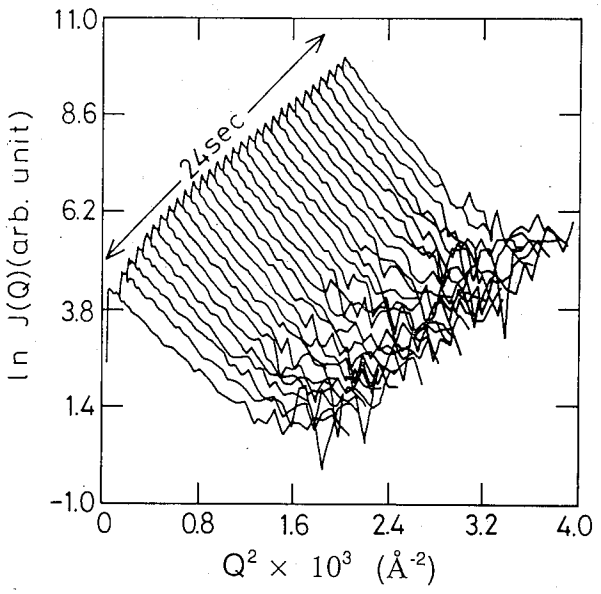

Fig. 4 A time course of Guinier plots evaluated from the results of temperature-jump time-resolved small-angle X-ray scattering experiments: 12.0 mg TMV protein ml^{-1} in 100 mM phosphate buffer at pH 7.2 and a T-jump from 5.0°C to 25.0°C. A total counting time for each curve was 16 sec (20 times of accumulation by 0.8 sec shot).

plotted against time in 100 mM phosphate buffer (pH 7.2) at a temperature-jump from 5.0°C to 25.0°C (a) for 5.0 mg TMVP ml^{-1} and (b) for 12.0 mg TMVP ml^{-1}. The $R_{g,z}$ value of the final frame evaluated for each experimental run was found to be consistent with that of the static measurement[10] under the corresponding condition. Generally, the assembly process is accelerated with increasing protein concentration and/or buffer concentration (ionic strength). The most prominent factor affecting the assembly is the temperature gap in the jump; the larger the gap, the more rapidly the association proceeds.

The initial values of the z-averaged radii of gyration in each experiment were found larger than 58 Å as seen from Table 1 as $R_{g,z}$ at t=0. This value of 58 Å lies between the $R_{g,z}$ values of A-protein (25.2 Å) and of 20S double-layered disk (66.5 Å)[10], suggesting that the TMVP assembly initiated by the temperature jump starts from an initial state where A-protein and 20S disk are in equilibrium and coexist in the solution.

The time course of the $R_{g,z}$ change of TMVP aggregation obtained from the T-J SAXS experiments can be applied to solving eq.(2) with the constraint of eq.(3) for determination of K_1, k_{+2} and k_{-2} in the TMVP aggregation reaction initiated by T-J under the condition investigated. The resulted values of the kinetic parameters are shown in Table 1. In Fig. 5, the plots of the calculated radii of gyration with estimated rate constants from eq.(4) are shown as a function of time by a continuous line and are compared with the experimentally estimated values of $R_{g,z}$. An agreement is excellent.

DISCUSSION

Analysis can be made by fitting the calculated molecular weight (eq.(5)) to the observed one. Since the absolute scattered intensity is not available in the present

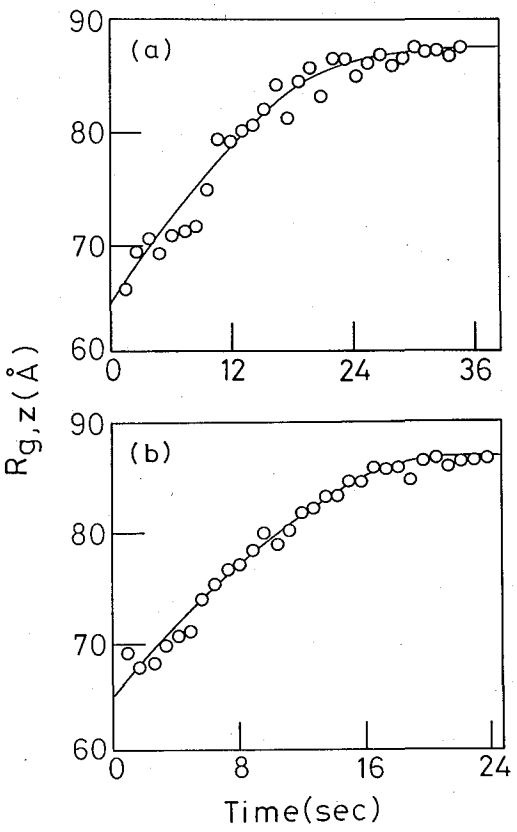

Fig. 5 Examples of the comparison of the experimental $R_{g,z}(t)$ with the calculated result (continuous line) of the analysis followed by the procedure described in the text. Here the T-Jump from 5.0°C to 25.0°C was observed by SAXS from TMV protein in 100mM phosphate buffer (pH 7.2) with the protein concentration (a) of 5.0 $mgml^{-1}$ and (b) of 12.0 mg/ml.

Table 1 Kinetic parameters evaluated for the TMVP assembly process

Temp. jump	Protein concn(mg/ml)	Buffer concn(mM)	$R_{g,z}$(Å) at t=0	$R_{g,z}$(Å) at t=∞	log K_1	$k_{+2}\times10^{-4}$ ($M^{-1}s^{-1}$)	$k_{-2}\times10^{-1}$ (s^{-1})
20°C	5.0	50		62.2			
	12.0	50	60.8	66.2	28.32	0.0857	0.1457
	5.0	100	61.8	77.4	32.03	0.4984	0.1035
5°C	12.0	100	62.0	78.5	28.12	1.2719	0.3914
25°C	5.0	50	60.9	71.5	31.22	1.4894	0.3156
	12.0	50	62.6	75.4	29.09	0.8423	0.3530
	5.0	100	63.4	90.0	32.89	8.8512	0.5361
5°C	12.0	100	65.2	91.3	31.82	3.5760	0.5203

system to evaluate the molecular weight directly, we can only know the relative molecular weight at each time frame with respect to the initial molecular weight. Accordingly the present analysis was made by fitting the radius of gyration rather than the molecular weight. The calculated relative weight average molecular weight from eq.(5) with respect to the initial molecular weight, however, was found in good agreement with the observed ratio of $J(0)/c$ to its initial value at each time frame and

confirmed the consistency of the present analysis. Considering a good agreement of two independent parameters $R_{g,z}(t)$ and $M_w(t)/M_w(0)$ with those observed in the time-course, the proposition seems reasonable that the TMVP aggregation process could be described in terms of the two-stage aggregation of TMVP with an equilibrium constant K_1, and two rate constants k_{+2} and k_{-2} for association and dissociation of disks, respectively. That is, the TMVP assembly proceeds in two steps of: (1) the aggregation of A-protein into double-layered disks and (2) the stacking of double-layered disks.

The k_{+2} values of different protein concentrations in the same ionic strength are in fair agreement with each other, as are the k_{-2} values as expected from kinetic theory. The k_{+2} value which was estimated as the order of 10^4 M^{-1} s^{-1} in the present system, is lower than that expected for normal diffusion-controlled association reactions, and falls in the lowest range of protein-protein interaction systems (10^8 to 10^4 M^{-1} s^{-1}; Koren & Hammes[14]). This result may suggest that the rod elongation of TMVP is due to disk stacking, which would not proceed so rapidly as forming oligomers which consist of only a small number of subunits.

The analytical procedure described in this paper could be applied to solving the kinetic parameters of association-dissociation reaction when the system is observed by time-resolved small-angle scattering in the combination with the temperature-jump or stopped-flow technique. As small-angle scattering evaluates a number of physical properties such as the radius of gyration, molecular weight, and the distance distribution function, the mechanism of dynamic reactions can be analyzed quantitatively in terms of the structural change.

This work was performed under the approval of the Photon Factory Programme Advisory Committee (Proposal nos. 84-003 and 86-026).

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