Measurement of the rotational behaviour of virus particles during adsorption to the host cell surface

M.E. Bayer, I. Morrison+ and R. Cherry+

Institute for Cancer Research, Philadelphia, PA 19111, USA and +*Department of Chemistry, University of Essex, Colchester C043SQ, England*

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The rotational diffusion of bacteriophage ε15 was measured before and after virus adsorption to outer membrane vesicles of the host *Salmonella anatum*. The virus capsid was labeled with eosin isothiocyanate, and the decay of transient dichroism following dye excitation by pulses of plane-polarized light was measured. From the data, the rotational diffusion constant of the unadsorbed virion and its hydrodynamic diameter were estimated and found to be consistent with electron microscopic measurements of the capsid dimensions. Addition of outer membrane vesicles of *S. anatum* to the virus suspension revealed the immobilization of the virus particles on the membrane surface.

Rotational diffusion *Virus adsorption* *Membrane vesicle* *Transient dichroism* *Hydrodynamic diameter* *Virus immobilization*

1. INTRODUCTION

We report a method that demonstrates changes in the rotational diffusion of relatively large molecular assemblies, such as those accompanying the adsorption of virus particles onto cell surfaces. The method provides data that are independent of measurements conventionally employed in determinations of adsorption kinetics [1–3], especially those used for the assessment of the adsorption of virus particles [4]. The method takes advantage of the long lifetime of the transient dichroism of the fluorescence dye eosin, which makes it possible to follow the polarization decay of relatively large, thus slowly tumbling, particles such as viruses. We studied the adsorption of bacteriophage ε15 and found that the difference between the rotational diffusion of unadsorbed and adsorbed virions is large enough to recommend this technique as a tool for adsorption studies of viruses.

Virus infections involve reversible and irreversible stages [1,4]. Virus translocations have been shown to occur during the early reversible stage: the virus particle moves laterally while it penetrates cell surface layers such as lipopolysaccharides and capsule polysaccharides [5]. We report the measurement of movements of the freely diffusing virus and those of the virus during the later stage of adsorption, after it has become firmly attached. We measured the change in the decay of transient adsorption dichroism of eosin-labeled phage ε15 before and after adsorption and found that (1) the hydrodynamic diameter of the unattached virion is in agreement with size determinations by electron microscopy, and (2) the adsorbed virus particle is practically immobilized on the cell surface.

2. MATERIALS AND METHODS

2.1. *Virus preparation and labeling*

Phage ε15 was prepared from lysates of *Salmonella anatum* and purified in cesium chloride gradients, as previously described [4]. For the labeling and adsorption studies we used only virus preparations that were less than 2 wk old. Virus was stored in M9 [2] containing 1% L-broth [5]. L-

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broth showed significant fluorescence at the wavelength needed and could not be used at full strength. Since we needed to keep the amount of free DNA (discharged from inactivated virions) to a minimum, we tested a variety of storage solutions with little or no measurable fluorescence of their own for their effect on phage viability (= plaque-forming units). Virus suspensions were stored at 4°C in media of pH 7.2 to 7.5: 75% of the phage was inactivated in 24 h in 0.1 M phosphate buffer, 85% in M9. With both media the titer dropped to a few percent within 7 days. However, in L-broth the plaque-forming titer remained unchanged for several weeks. We found that the protective capability of L-broth is maintained at 20-fold dilution in M9, so we kept the virus in this mixture of M9 and L-broth.

For staining, the purified phage suspension (titers between 1 and 3×10^{12}/ml) was added 1:1 (v/v) to a mixture of equal volumes of M9 and 0.1 M $NaHCO_3$ with a pH of 7.4. 200 μg eosin isothiocyanate (Molecular Probes, Junction City, OR) per mg virus protein were added. Protein was determined according to [7]. After incubating for 2 h at 22°C in the dark, the virus suspension was sedimented in polycarbonate tubes at 200000 $\times$ g for 2.5 h at 4°C. The supernatant was decanted, the tube gently flushed twice with M9 and the pellet either resuspended in 1 ml M9 by agitation for 10 min, or the pellet was first cut out of the tube and placed in 1 ml of M9. The latter method reduced the background caused by unconjugated dye. In 4 experiments, more than 60% of the virus titer was recovered after the labeling procedure.

2.2. *Cell membrane preparation*

S. anatum was grown in exponential phase in L-broth at 37°C to 2×10^8 cells/ml. The culture was rapidly chilled, and the cells were sedimented at 4000 $\times$ g, resuspended in M9 and disrupted in a French press cell [6]. The membrane vesicles were separated in a sucrose step gradient and dialyzed against M9. The outer membrane (OM) fractions (= fraction 3, see [6]) were used in the adsorption studies. Immuno-ferritin labeling of the lipopolysaccharide (type E1) with anti-E1 globulin–ferritin conjugate revealed that more than 80% of the vesicles were right-side-out. The protein concentration of the OM preparations varied between 1 and 6 mg/ml.

2.3. *Preparation for spectroscopy*

A 200-μl virus suspension was added to 200 μl buffer mixture. Virus as well as vesicle preparations were flushed with argon for several minutes at 0°C to reduce bleaching caused by oxygen and placed in cuvettes cooled to 10°C, and again flushed with argon. For the adsorption experiments, 200 μl of vesicles were added to the virus suspension within the cuvette, and argon again flushed over the liquid surface.

The instrumentation for spectroscopy and data treatment has been described in detail [8,9]. Briefly, rotation was measured by observing transient dichroism of ground-state depletion signals arising from excitation of the eosin probe by a linearly polarized light pulse from a NA-YAG laser (J.K. Lasers). Excitation was at 535 nm and absorbance changes were recorded at 515 nm for light polarized parallel and perpendicular to the plane of polarization of the exciting flash. Each signal is comprised of 100 data points; typically 512 signals were collected and averaged in a Datalab DL 102A signal averager. Data were analysed by calculating the absorption anisotropy $r(t)$ given by

$$r(t) = \frac{A_{\parallel}(t) - A_{\perp}(t)}{A_{\parallel}(t) + 2A_{\perp}(t)} \quad (1)$$

where $A_{\parallel}(t)$ and $A_{\perp}(t)$ are the absorbance changes as a function of time t for light polarized parallel and perpendicular, respectively, to the plane of polarization of the exciting flash. For isotropic rotation, the decay of anisotropy is given by

$$r(t) = r_o \exp(-t/\phi_c) \quad (2)$$

where r_o is the anisotropy at time $t = 0$ and ϕ_c is the rotational correlation time. For a sphere of radius a in a medium of viscosity η

$$\phi_c = \frac{4\pi a^3 \eta}{3kT} \quad (3)$$

The anisotropy decays for the free virus were fitted to eq.(2) using a least-squares regression. The values of ϕ_c so obtained is then inserted into eq.(3) to calculate the effective radius of the virus.

For electron microscopy, virus and vesicle preparations were stained in 0.2% uranylacetate. Some specimens were fixed with 2% formaldehyde in M9 prior to staining. For positive staining, virus was first added to carbon films, then exposed to the uranyl acetate for 2–5 min, and briefly washed

in distilled water. Micrographs were taken in either a Siemens 101 or a Philips 400.

3. RESULTS

3.1. *Bacteriophage ϵ15 in solution*

3.1.1. Electron microscopy

The virus is composed of an icosahedral capsid with an adsorption organelle attached to one of its vertices. The virus diameter was found to be 65 ± 4 nm in negatively-stained preparations. The 6 short 'pins' of the adsorption apparatus add 8 to 10 nm to the length of one axis of the capsid.

3.1.2. Spectroscopy

Fig.1 shows the anisotropy (r) plotted against the time in μs. The decay of $r(t)$ from 0–200 μs corresponds to Brownian rotation of the virus in the buffer. A repeat of 512 flashes with the same material in the cuvette revealed increasing scatter of the data points, probably due to some bleaching of the dye during the measurements.

For the data, the rotational correlation time of the virion was determined to be 43 ± 6 μs. From the value the hydrodynamic diameter was calculated from eq.(3) to be 63 ± 3 nm.

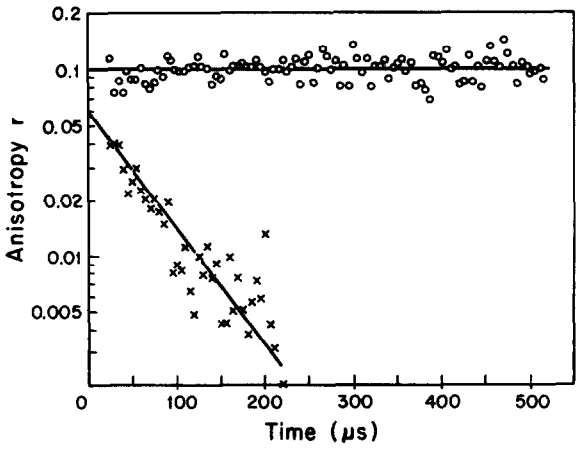

Fig.1. Anisotropy (r) is plotted against time (μs) for the eosin-labeled phage 15. (x) Unadsorbed virus. The data points are from 512 flashes. Points beyond 0.2 ms are the mean of 4 neighbours. A repeat of 512 flashes caused higher scatter (not shown). (○) Virus adsorbed to outer membrane vesicles. The anisotropy remains constant with time.

We observed that only recently prepared virus can be used for the experiments, since the virus becomes unstable after labeling. Storage longer than 12 h was accompanied with significant loss of titer and release of DNA. The electron microscope shows (fig.2) virus particles 24 h after eosin labeling. DNA strands surround each virion. We therefore performed our experiments immediately after labeling.

The amount of eosin bound per virion was estimated from eosin standards and the absorption of the labeled virus preparations. We obtained an eosin content of 5×10^{-13} μg per virus capsid. 60–70% of the viruses measured had retained their viability after labeling. We estimated further, that about 450 eosin molecules were attached to each virus capsid, and that these dye molecules might occupy less than 10% of the capsid surface.

3.2. *Membrane vesicles*

We used outer membrane vesicles rather than whole cells in order to reduce light scattering. The vesicle diameter exceeded that of the phage by more than 5-fold, many vesicles being considerably larger than that. The density of the vesicles placed in the cuvette at the desired concentration caused a slight visible turbidity. The immobilization of the virus particle by the vesicles can be seen in fig.1 (upper plot) from the lack of decay of the anisotropy. Electron microscopic examination of

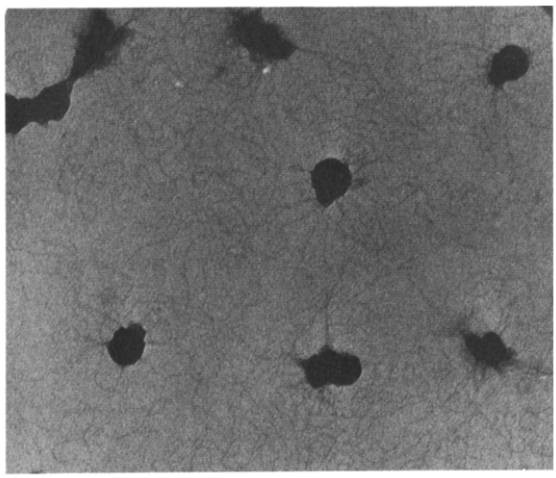

Fig.2. Bacteriophage ϵ15, prepared 24 h after eosin-labeling. The electron micrograph shows DNA strands surrounding the virus particles (113330 ×).

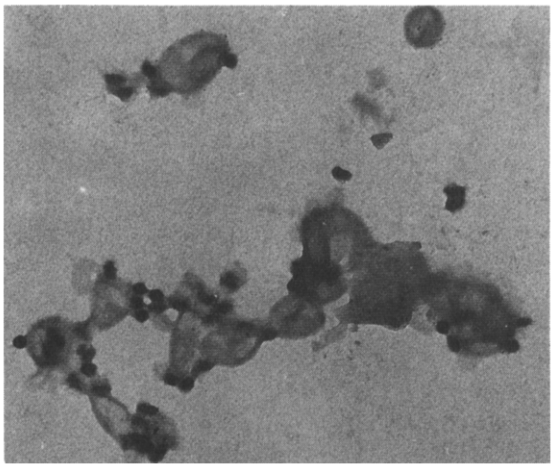

Fig.3. Bacteriophage ϵ15 after 10 min adsorption to outer membrane vesicles, at 10°C. The specimen was prepared shortly before spectroscopy. Positive stain in 0.5% uranylacetate (44000 ×).

the contents of the cuvette before (fig.3) and after the experiment revealed that the virus particles remained attached to the vesicles. We also estimated from the electron micrographs, that at most 12% of the vesicle surface was occupied by phage particles.

4. DISCUSSION

We have demonstrated that the rotational motion of a relatively large macromolecular assembly, such as a bacteriophage, can be determined by measurement of the absorption anisotropy of covalently linked eosin label. Experiments of this nature have also been previously performed with ribosomes [10]. The rotational diffusion constant and the hydrodynamic diameter derived from it are in good agreement with the electron microscopic measurements. We found that the labeling procedure induces a considerable degree of instability of the otherwise rather stable virus capsid. Therefore, the labeled virus had to be used as soon as possible, most certainly within a time span of about 8–12 h. At longer periods, the DNA leaking from the virus capsids will interfere with the diffusion of the virions.

The attachment of the virion to the cell surface was strong and did not allow for significant degrees of rotation. Since the vesicles are much larger than the phage, the rotation due to tumbling of the membrane vesicles themselves is too slow to be detected on the time scale of the experiment. The method describes the end stage in the adsorption process. Normally at this stage and at temperatures above 21°C, the DNA would be released by this type of phage (unpublished). Further development of the method should also allow us to study much earlier steps in virus adsorption to host cell surfaces. The technique provides a valuable tool for the measurement of interactions of macromolecules with receptor-containing surfaces.

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