Binding of Intercalating and Groove-Binding Cyanine Dyes to Bacteriophage T5

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The interaction between four related cyanine dyes and bacteriophage T5 is investigated with fluorescence and absorption spectroscopy. The dyes, which differ in size, charge, and mode of DNA-binding, penetrate the capsid and bind the DNA inside. The rate of association decreases progressively with increasing dye size, from a few minutes for YO to more than 50 h for YOYO (at 37 °C). The relative affinity for the phage DNA is a factor of about 0.2 lower than for the same T5-DNA when free in solution. Comparison of groove-bound BOXTO-PRO and intercalating YO-PRO shows that the reduced affinity is not due to DNA extension but perhaps influenced by competition with other cationic DNA-binding agents inside the capsid. Although, the extent of dye binding to the phages decreases with increasing external ionic strength, the affinity relative to free DNA increases, which indicates a comparatively weak screening of electrostatic interactions inside the phage. The rate of binding increases with increasing ionic strength, reflecting an increase in effective pore size of the capsid as electrostatic interactions are screened and/or a faster diffusion of the dye through the DNA matrix inside the capsid as the DNA affinity is reduced. A combination of electron microscopy, light scattering, and linear dichroism show that the phages are intact after YO-PRO binding, whereas a small degree of capsid rupture cannot be excluded with BOXTO-PRO.

Introduction

During the past decade there has been a growing interest in bacteriophages within the fields of molecular biology, biochemistry, and biophysics, mainly due to an increased number of cloned phage proteins and receptors, the development of single molecule approaches, and improvements of structural tools such as 3D reconstruction from electron micrographs. Image reconstruction has been used to investigate the structure of several phages,[1−3] as well as the complex between the T5 phage and its *Escherichia coli* receptor FhuA inserted into a liposome membrane.[4] Single-phage experiments[5,6] were used to study the process when bacteriophage ϕ29 packages its DNA into the capsid, against pressures as large as 60 atm. In vitro packaging was used to analyze mutations in the T4 DNA packaging ATPase.[7] These types of investigations demonstrate how DNA packagings in bacteriophages are becoming model systems for studies of molecular motors as mechanochemical systems.

The DNA packaging is just the first step of the biochemical cycle of bacteriophages, however, and it is important to understand the subsequent ejection process to the same molecular degree. In vitro systems have been used to study DNA ejection, either by dissolving the bacterial receptor in bulk solution or by introducing the receptor into liposomes. Fluorescence measurements were used to study the injection of λ-DNA into liposomes modified with the receptor LamB and containing the dye ethidium bromide for DNA detection.[8] The ejection of T5 DNA into bulk solution containing the solubilized FhuA receptor was similarly studied by fluorescence spectroscopy using the DNA probe YO-PRO[9] and by light scattering.[10] The same in vitro T5 system was used to study DNA ejection from individual phages by use of fluorescence microscopy,[11] which showed that the ejection from individual phages takes places in a stepwise fashion. Recently, in vitro studies showed that the DNA ejection ability of the λ-phage was suppressed as the external osmotic pressure was increased by addition of polyethylene glycol.[12] Similarly, the driving force for ejection of phage T5 DNA was decreased by addition of tetravalent cation spermine, which penetrates the capsid and bind to the DNA inside and thereby decrease the internal pressure.[10,12]

Such in vitro studies indicate that DNA ejection is controlled by the chemical potential of the DNA inside the capsid. It is therefore important to characterize the physicochemical environment inside bacteriophage capsids, to better understand the molecular mechanism of DNA ejection. An important aspect is then the permeability of the capsid wall, because experiments with both small ions[13] and comparatively large spermine[10,12] indicate that phage capsids may be open to external molecular influences. Characterization of the ionic environment inside the capsid is also important, for instance because of the electrostatic contribution to the repulsion between closely packed DNA strands.[7]

Capsid penetration can be probed in a direct fashion by using DNA-binding dyes, because their spectral response upon binding to the DNA inside the capsid can be used to monitor rates of diffusion through the capsid wall. On the basis of this approach, studies of ethidium bromide (EB) with phage T7 (reference 13) and of proflavine (PF) with phage T2 and T4[14] showed that both dyes are capable of penetrating the corresponding capsids and bind to the phage DNA on the time scale of tens of minutes and hours, respectively, at 37 °C. In both systems the binding constant to the phages was found to be almost 1 order of magnitude smaller than for the same DNA when free in solution.[15,16] The decrease in affinity was suggested to be caused

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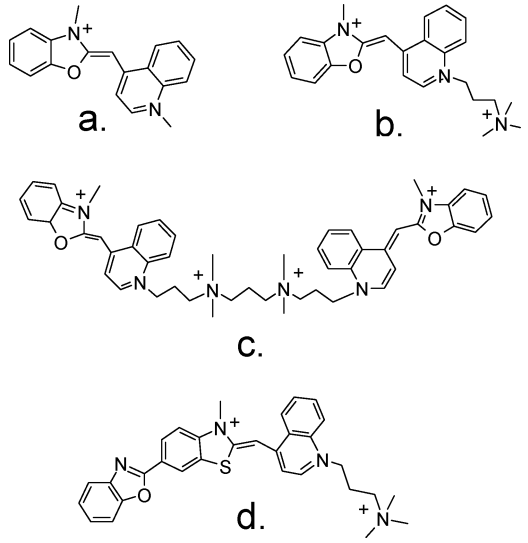

Figure 1. Structure of the dyes used in this study: (a) YO; (b) YO-PRO; (c) YOYO; (d) BOXTO-PRO.

either by competition from cationic molecules such as polyamines or by the fact that the binding of EB and PF to the tightly packaged DNA is restrained because these intercalators have to extend the DNA helix.

In this study the capsid permeability of phage T5 is investigated systematically, by using three DNA-binding cyanine dyes YO, YO-PRO, and YOYO (Figure 1) which differ in size, while their spectroscopic response upon DNA-binding is the same.[17] The ionic environment of phage T5 is probed by exploiting that this "homologue" series of YO dyes covers a range of dye charges between +1 and +4 and by varying the ionic strength of the external solution. Finally, we investigate the effect of DNA extension on the effective phage-binding constant by comparing YO-PRO and BOXTO-PRO. These dyes have the same charge and similar size but differ in the mode of DNA-binding: YO-PRO intercalates and subsequently extends and unwinds the DNA,[18−20] whereas BOXTO-PRO is groove-bound[21] and does not perturb the helix to same extent.[22]

Materials and Methods

Materials. Wild-type phage T5 (DNA 121.7 kbp)[23] was prepared from *E. coli* strain Fsuβ+ by standard protocols and purified by polyethylene glycol precipitation followed by CsCl gradient centrifugation as previously described.[24] Stock solutions of phages were stored at 8 °C in Tris-HCl phage buffer (1 mM, pH 7.5), containing NaCl (150 mM), $CaCl_2$ (1 mM), and $MgCl_2$ (1 mM). The stock phage concentration was 1.5×10^{13} phage particles/mL, as determined by complete extraction of the phage DNA (see below), which was subsequently quantified by YO-PRO titration and comparison with DNA standards of known concentration. All measurements were performed at 37 °C, after dilution of the stock solutions with the phage buffer. Proteinase K, T5 DNA, T7 DNA (39 kbp), and agarose were obtained from Sigma-Aldrich, and λ-DNA (48 kbp) and HindIII-digest of λ-DNA were obtained from Amersham, Piscataway, NJ. BOXTO-PRO and YO were gifts from G. Westman and Per Lincoln, respectively (Chalmers University of Technology, Gothenburg, Sweden), whereas YO-PRO and YOYO were obtained from VWR. The concentrations of YO, YO-PRO, and YOYO were determined by absorption measurements by using the following extinction coefficients in aqueous solution: 66 000 M^{-1} cm^{-1} (482 nm) for YO and YO-PRO; 96 100 M^{-1} cm^{-1} (457 nm) for YOYO. With BOXTO-PRO a methanol protocol[21] was used to avoid effects on the absorption from dye dimerization in aqueous solution.

Extraction of T5 DNA. T5 DNA was extracted by incubating the phages (2.2×10^{11} phage/mL) at 56 °C with proteinase K (5 μg/μL) in aqueous solution. SDS was avoided since it may interfere with subsequent dye binding. The digestion was monitored (between 10 min and 24 h) by electrophoretic quantification on 1% agarose gel in TBE buffer (50 mM Tris, 50 mM sodium borate, 1.25 mM EDTA, pH = 8.2), after field inversion electrophoresis at 7.5 V/cm with $T_+ = 3$ s and $T_- = 1$ s. The DNA zones were visualized after ethidium bromide staining by scanning the gel in a Molecular Dynamics FluoroImager gel scanner 595 (excitation at 514 and 610 nm long-pass emission). The amount of extracted DNA was quantified by use of the software ImageQuant (GE Healthcare). Complete extraction of the phage DNA was achieved after 6 h of incubation (Supporting Information). Throughout this study, the DNA obtained by the 6 h protocol will be referred to as extracted T5 DNA, to distinguish it from the DNA enclosed in the capsid. Notably, in the native phage samples about 2.5% of the total T5 DNA was present as free DNA (released during phage preparation), as determined by fluorescence measurements (see below).

Fluorescence Spectroscopy. The interaction between the cyanine dyes (Figure 1) and bacteriophage T5 was studied at 37 °C. For fluorescence studies the samples contained 9.6×10^9 phage particles/mL (1.92 μM bp). The concentrations of YO, YO-PRO, and BOXTO-PRO were 1.22 μM, while the concentration of YOYO was 0.52 μM. Those mixing ratios (dye/bp) were chosen to ensure a dye excess of 30% over the number of binding site. The binding of the dyes to the phages was followed by the increase in fluorescence intensity with time, using a SPEX fluorolog τ-2 spectrofluorometer or a Spex Fluorolog τ-3 spectrofluorometer (Horiba Jobin Yvon Inc.) The phage-dye samples were excited at the absorption maximum in the visible region, and the fluorescence was measured at the wavelength of maximum emission (523 and 542 nm, respectively, for BOXTO-PRO, 476 and 507 nm for YO, 491 and 509 nm for YO-PRO, and 491 and 509 nm for YOYO). Due to the overall slow rate of binding, possible bleaching of the dyes was avoided by using intermittent illumination of the samples (with the dark time typically set to 30 s and illumination time to 1 s), except for YO where the binding occurs in minutes. The association kinetics was best described by a double exponential fit of the emission data (three exponentials did not improve the fits).

The same types of dye-association time profiles were measured with extracted T5 DNA. The relative amount of phage-bound dye was then determined by comparing the final (steady-state) fluorescence intensities measured with the phages and with extracted T5 DNA at the same basepair concentration, using the same dye concentration and identical settings on the fluorometer.

Absorption, excitation, and emission spectra were recorded at suitable time intervals during the association (on a parallel sample which was incubated in an identical manner), using a Cary 4000 Bio-UV−vis spectrophotometer and a Cary Eclipse spectrofluorometer (Varian).

Electron Microscopy. The phage samples (2−5 μL) were plunge-freezed in a controlled environment vitrification system (CEVS)[25] with high humidity in the chamber and liquid ethane (−180 °C) as cryogen. Lacey carbon filmed Cu grids were used to form a thin film of amorphous ice (100−300 nm) covering the holes in the carbon film. The grids were transferred to a

cryo-holder (Oxford CT3500) and were kept below −180 °C at every step. Microscopy was performed using a Philips CM120 BioTWIN TEM operated at 120 kV, and micrographs (700−800 nm underfocus) were taken using a Gatan 791 cooled multiscan CCD camera. Samples for cryo-transmission electron microscopy (cryo-TEM) contained either 1×10^{12} or 5×10^{12} phage particles/mL. Images of native phages, proteinase-digested phages (2 h), and phages which had been incubated with YO-PRO or BOXTO-PRO (63 μM) for 3 and 24 h, respectively (to reach binding equilibrium), were collected.

Light Scattering. Dynamic light scattering (DLS) at a wavelength of 632.8 nm was measured using an ALV CGS-8F DLS/SLS-5022F instrument (ALV, Langen, Germany), equipped with an ALV-6010/160 correlator and dual APD detectors. The scattering angle was maintained at 90°, and the measurements were performed at 37 °C. If nothing else is stated, the samples contained 6.0×10^{10} phage particles/mL (~12 μM basepair) and the mixing ratio dye/bp was 1/1. The autocorrelation curves were obtained as an average over typically 5−10 consecutive measurements. The diffusion coefficient was determined from the initial decay of the autocorrelation function by a second-order cumulant analysis, as described previously.[26] Since the volume fraction of phages was 5×10^{-5}, the samples could be considered to be hydrodynamically dilute, and a hydrodynamic radius was extracted by assuming spherical phage geometry. In the same experiment the average intensity of the scattered light was measured (static light scattering SLS), which reflects the amount of remaining DNA inside the capsid.[10]

In some experiments, proteinase (5.0×10^{-3} g/mL) was present in the sample, to monitor the effect of capsid damage and DNA release on the diffusion coefficient and scattered intensity. Even if the DNA is released from all the phages into the surrounding solution, it can be assumed to have negligible effect on the hydrodynamic behavior of the phages. Similarly, the dissolved proteinase can be assumed to have negligible effect on the sample viscosity. The scattered intensity from proteinase K molecules, from released DNA in the solution[10] and from the dye molecules, was negligible compared to scattered intensity from the phages.

Linear Polarized Spectroscopy. Linear dichroism (LD) is the difference in absorption of light which is linearly polarized perpendicular and parallel to a macroscopic orientation axis, in our case the direction of the flow in a Couette cell.[27] Potential release of DNA from the phages after dye-binding LD was monitored at 37 °C by using a Jasco-720 spectropolarimeter (Jasco), equipped with a Couette cell with an outer rotating cylinder. The mixing ratio was the same as during the fluorescence measurements, but all concentrations were increased by a factor of 7 ([DNA bp] = 13 μM, [dye] = 8.5 μM).

Results

Association of Dyes with Bacteriophage T5. *Fluorescence Intensity.* The binding of YO, YO-PRO, YOYO, and BOXTO-PRO (Figure 1) to phage T5 was followed by monitoring the fluorescence enhancement with time. Figure 2 (main parts) shows the association time profiles of the dyes to the phages, whereas the insets show the binding of the corresponding dye to the same amount of T5 DNA free in solution ("extracted T5 DNA"). It is seen that dye association to the T5 phages is much slower than to the extracted DNA, except for YO where the two rates are comparable. The initial decrease in intensity seen with extracted DNA is due to a temperature adjustment. If not otherwise stated, the results were obtained in phage buffer with 150 mM NaCl.

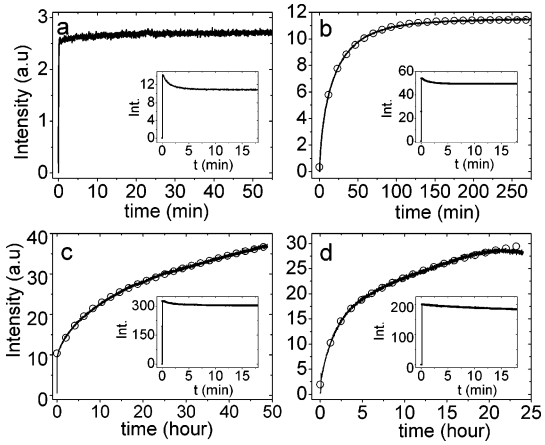

Figure 2. Association of (a) YO, (b) YO-PRO, (c) YOYO, and (d) BOXTO-PRO with bacteriophage T5 monitored by the increase in fluorescence intensity (au = arbitrary units) with time. The association profiles were fitted to double exponentials (white circles). The insets show the corresponding time profiles when the dyes are added to extracted T5 DNA under identical conditions. [DNA bp] = 1.92 μM, [YO] = [YO-PRO] = [BOXTO-PRO] = 1.22 μM, and [YOYO] = 0.52 μM. NaCl concentration 150 mM.

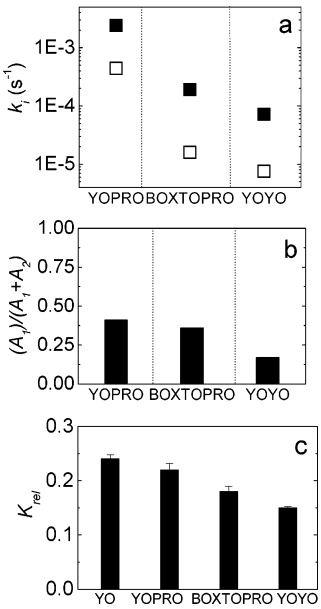

Figure 3. (a) Rate constants (k_1, solid squares; k_2, open squares) and (b) relative amplitudes of the fast processes for binding to phage T5, obtained by fitting data in Figure 2b−d to eq 1. YO association was too fast to obtain good fits. (c) Relative affinity constants (with error bars) calculated as the ratio of the final intensities measured with phages and extracted DNA.

The association rate to the phages decreases strongly with size of the dyes: YO binds within 1 min whereas YOYO has not reached steady state even after 50 h. YO-PRO (Figure 2b) and BOXTO-PRO (Figure 2d) are seen to have association rates between these two extremes. The association profiles could be described by double exponentials (with amplitudes A_i and corresponding rate constants k_i)

$$I(t) = I_o + A_1(1 - e^{-k_1 t}) + A_2(1 - e^{-k_2 t}) \qquad (1)$$

except with YO where the association is too fast to be monitored by our approach of manual mixing, as was also the case with extracted DNA for all four dyes. Figure 3 shows the fitted rate constants k_1 and k_2 for YO-PRO, YOYO, and BOXTO-PRO (Figure 3a), as well as the relative amplitude A_1 for the fast

TABLE 1: Association of Cyanine Dyes with Phage T5 at 150 mM NaCl

param	YO	YO-PRO	YOYO	BOXTO-PRO
K_1 (s^{-1})[a]	nd[b]	2.4×10^{-3}	7.2×10^{-5}	1.9×10^{-4}
K_2 (s^{-1})[a]	nd[b]	4.4×10^{-4}	7.6×10^{-6}	1.6×10^{-5}
$A_1/(A_1+A_2)$[a]	nd[b]	0.41	0.17	0.36
$A_1 + A_2$ (au)[c]	2.4	10	37	34
K_{rel}[d]	0.24 ± 0.01	0.22 ± 0.01	0.15	0.18 ± 0.01

[a] Rate constants (k_1, k_2) and amplitudes (A_1, A_2) obtained from fits to eq 1. [b] Not determined because the association was too fast. [c] Obtained from the final intensity, after correction for free DNA in the phage sample (see text) (au = arbitrary units). [d] Ratio between the final intensities with phages and with extracted T5-DNA, after correction for free DNA in the phage sample (average values and standard errors of n measurements, $n = 2-3$).

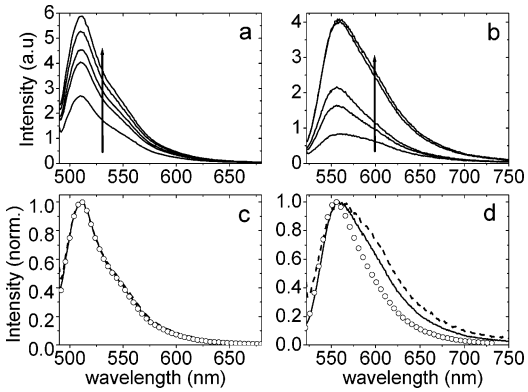

Figure 4. Emission spectra during association of (a) YO-PRO and (b) BOXTO-PRO to phage T5, measured after 10, 40, 70, 120, and 210 min and after 5, 45, and 90 min and 16 and 22 h, respectively (arrows indicate increasing time). (c, d) Corresponding normalized spectra of the dyes with phages (the first and last spectra in (a) and (b), dashed and solid lines, respectively) and corresponding spectra of the dyes bound to extracted T5 DNA (circles). [DNA bp] = 1.92 μM, and [dye] = 1.22 μM.

process (Figure 3b). (Numerical values are given in Table 1.) The I_o value corresponds to dye binding to free DNA, which is present as a result of the phage preparation. In the phage sample used here the fraction of free DNA was (2.5 ± 2)% of the total DNA, as calculated from the fitted I_o values. All intensities presented here have been corrected for the emission due to dye binding to this fraction of free DNA.

Figure 3c presents the relative dye affinity K_{rel} between phage T5 and extracted T5 DNA, obtained as the ratio of the final (corrected) emission intensities observed with phages and with extracted DNA. It is seen that the relative affinity is lower than unity for all the dyes and that K_{rel} decreases in the order YO, YO-PRO, BOXTO-PRO, and YOYO.

Fluorescence and Absorption Spectra. Figure 4 shows the emission spectra measured at specific times during the association of YO-PRO (a) and BOXTO-PRO (b) to phage T5, where the emission intensities are seen to increase with time in agreement with Figure 2.

The normalized emission spectra for YO-PRO (Figure 4c) are superimposable throughout the association, indicating that YO-PRO binds to the phages in the same manner during the process. The normalized spectra are also very similar to the corresponding spectrum of YO-PRO bound to extracted T5 DNA (open circles). This observation supports that YO-PRO binds to the DNA of the phages and furthermore in a manner similar to how it binds to the T5 DNA when free in solution. With BOXTO-PRO (Figure 4d) the emission spectra bound to the phages are initially red-shifted compared to that observed

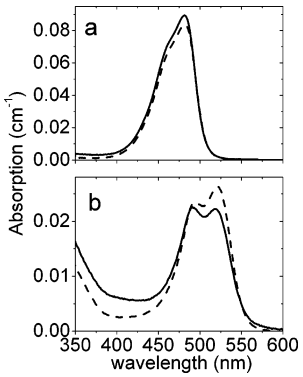

Figure 5. Absorption spectra measured after binding of (a) YO-PRO and (b) BOXTO-PRO to bacteriophage T5 (after 210 min and 22 h, respectively) (solid lines) and to extracted T5 DNA (dashed lines). [DNA bp] = 1.92 μM, and [dye] = 1.22 μM.

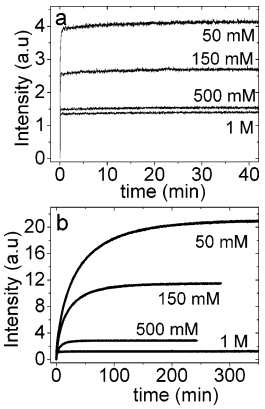

Figure 6. Association of (a) YO and (b) YO-PRO to T5 bacteriophage at indicated NaCl concentrations monitored by the increase in fluorescence intensity (au = arbitrary units) with time. [DNA bp] = 1.92 μM, and [dye] = 1.22 μM.

with extracted DNA, but they approach the latter in shape as the association to the phages proceeds. This observation suggests that BOXTO-PRO experiences a slightly different environment in its binding to DNA inside the capsid compared to how this dye binds to T5 DNA free in solution.

Figure 5 (solid) shows the corresponding absorption spectra of YO-PRO (a) and BOXTO-PRO (b) bound to phage T5 (after association is completed), together with the spectra for these dyes when bound to extracted T5-DNA (dashed). The two absorption spectra of YO-PRO are very similar, with a slightly lower intensity for the extracted T5 DNA suggesting a somewhat higher degree of binding to DNA free in solution than to phages (because YO-PRO binding is hypochromic[17]). Still, the absorption maximum is at 481 nm, which indicates that there is a substantial fraction of unbound dye.[17] With BOXTO-PRO both spectra exhibit a two-peak type of absorption spectrum, with the red-shifted peak being more prominent in the case of extracted DNA. These observations support[21] that a larger fraction of the dye is bound to the DNA when free in solution than when it is inside the capsid.

Effect of Ionic Strength. Figure 6 shows the association time profiles for YO (Figure 6a) and YO-PRO (Figure 6b) to phage T5 at various NaCl concentrations between 50 mM and 1 M. The association rate for YO-PRO is seen to increase significantly with increasing ionic strength, and the same trend is observable with YO, if only just barely. (Fitted rate constants at the various ionic strengths are given in Supporting Information.) Furthermore, it is seen that for both dyes the final emission intensity decreases as the NaCl concentration is increased.

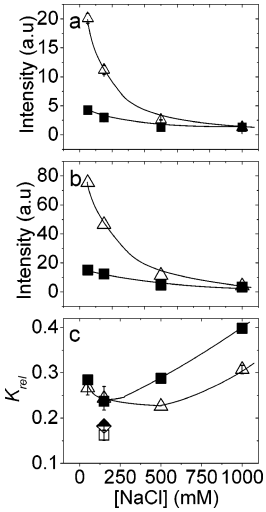

Figure 7. Final emission intensity versus NaCl concentration for YO-PRO and YO (open triangles and solid squares, respectively) bound to (a) phage T5 and (b) extracted T5 DNA. (c) Relative affinity, K_{rel}, of the dyes for phage T5 compared to extracted T5 DNA vs NaCl concentration, obtained as the ratio between intensities in the top and middle panels. The corresponding data for YOYO (open square) and BOXTO-PRO (half-filled diamond) at [NaCl] = 150 mM are included. (Error bars are shown when larger than the symbols; lines are only guides to the eye.) [DNA bp] = 1.92 μM, [YO] = [YO-PRO] = [BOXTO-PRO] = 1.22 μM, and [YOYO] = 0.52 μM.

Figure 7a shows the final fluorescence intensities for YO-PRO and YO binding to the phages vs NaCl concentration, and Figure 7b presents the same set of data for these dyes when binding to the extracted DNA. It is seen that, for a given type of dye, the intensity decreases with increasing ionic strength in a similar manner for phages and extracted DNA. In particular, we note that the intensities with YO and YO-PRO approach each other at the highest ionic strength. Comparison of Figure 7a,b also shows that, at a given ionic strength and dye type, the intensity with the phages is lower than with the extracted DNA. Figure 7c shows the relative affinity K_{rel} for the phages on the basis of the ratio between the intensities and also includes K_{rel} for BOXTO-PRO and YOYO at 150 mM NaCl (from Figure 3c). It is seen that K_{rel} is significantly lower than unity under all studied conditions, meaning that all dyes bind less strongly to the phages than free DNA at all studied ionic strengths. With YO and YO-PRO K_{rel} is seen to increase with increasing ionic strength; i.e., the difference in affinity between phages and extracted DNA decreases. Still, even with YO at 1 M NaCl, the relative phage affinity is only about 0.4.

If we turn to the association kinetics, Figure 8a shows the salt dependence of the association rate constants for YO-PRO binding to phage T5. (The numerical values are given in the Supporting Information.) It is seen that the rate constant for the fast process (squares) shows a substantial increase with increasing NaCl concentrations, whereas the slow process (triangles) is less affected by the ionic strength. Figure 8b shows how the relative amplitude of each process is affected by the salt concentration. The two processes contribute approximately equally at low salt concentration, but at the highest salt concentration (1 M NaCl), the faster of the two processes dominates the response.

Effect of Dye Binding on the Phage Status. One question in this study was how the integrity of the bacteriophages is affected by the dye binding, which was investigated by electron microscopy, light scattering, and linear dichroism.

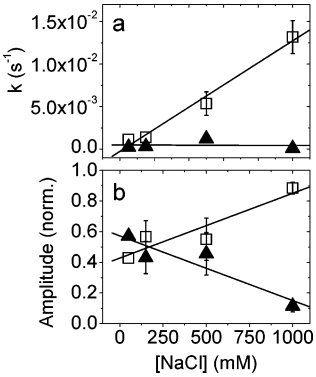

Figure 8. (**a**) Rate constants for YO-PRO association to bacteriophage DNA at varied NaCl concentrations (k_1 = open squares, k_2 = solid triangles). (b) Relative amplitudes for the processes at different salt concentrations. (Error bars are shown when larger than the symbols; lines are only guides to the eye.) [DNA bp] = 1.92 μM, and [YO-PRO] = 1.22 μM.

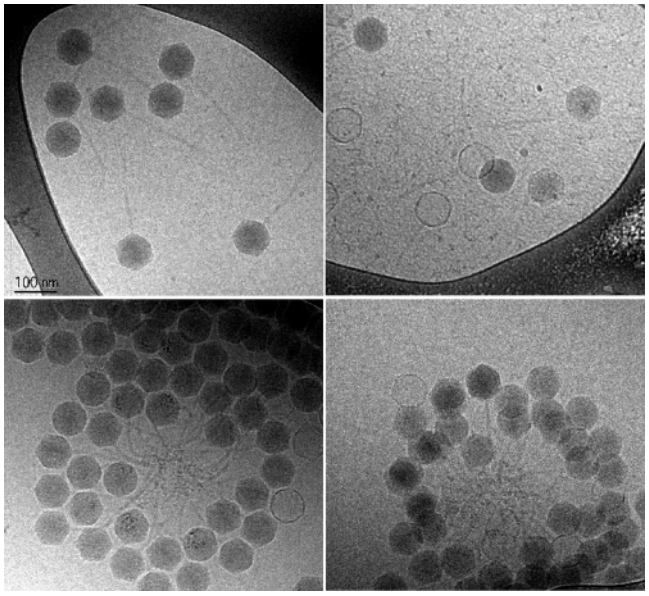

Figure 9. Cryo-transmission electron micrographs of bacteriophage T5: (a; upper left) native phages; (b; upper right) phages partially digested by proteinase K (2 h at 56 °C); (c; lower left) phages after association with YO-PRO (3 h at 37 °C); (d; lower right) phages after association with BOXTO-PRO (24 h at 37 °C). [NaCl] = 150 mM and [phage] = 5×10^{12} phage particles/mL, corresponding to [DNA bp] = 1 mM and [dye] = 63 μM (bar = 100 nm).

Cryo-Transmission Electron Microscopy. Figure 9 compares the cryo-TEM images of native phage sample (Figure 9a) with phages which were treated with proteinase (Figure 9b) and with phages after incubation with an excess of YO-PRO (Figure 9c) and BOXTO-PRO (Figure 9d). The dark capsids typically seen with native phages indicate that they contain DNA, whereas capsids with low contrast are empty. The proteinase-treated sample also contains phages with intermediate contrast, which indicates that those phages are partially filled with DNA. Phages incubated with YO-PRO or BOXTO-PRO form circular-shaped aggregates of mainly DNA-filled bacteriophages, with a few empty bacteriophages contributing. Such aggregates were not observed with the native phages or in the proteinase-treated sample.

Table 2 presents estimates of the number of full and empty phages in each sample based on approximately 30−50 microscopy pictures/sample. The fraction of empty phages was the same in the YO-PRO sample as in the native phage sample

TABLE 2: Status of Bacteriophage T5 (%), As Determined by Cryo-TEM

	native sample	proteinase treated[b]	YO-PRO[c]	BOXTO-PRO[d]
filled phages[a]	92	20	92	87

[a] Strong contrast due to DNA. [b] 2 h of incubation at 56 °C. [c] 3 h of incubation at 37 °C. [d] 24 h of incubation at 37 °C. The BOXTO-PRO values are more uncertain than the others, due to substantial dye-induced aggregation of the phages (see text). Many phages could not be evaluated individually, which may bias the statistics (either way).

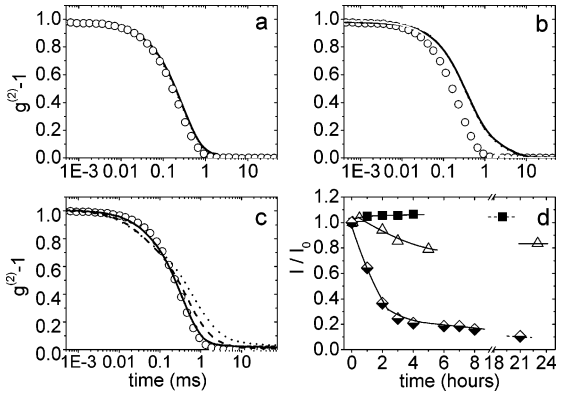

Figure 10. DLS measurements during association of (a) YO-PRO and (b) BOXTO-PRO to bacteriophages T5 after 5 min and 1 and 3 h (solid, dashed, and dotted lines) and after 1, 3, 5, and 24 h (black solid, dashed, dotted, and gray lines), respectively. (c) DLS measured for phages treated by proteinase K at 56 °C after 1, 3, and 6 h (solid, dashed, and dotted lines). Native phage samples are included in all panels (open circles). (d) Scattered intensity (normalized to the intensity of native phages) for phages incubated with YO-PRO (solid squares), BOXTO-PRO (open triangles) and proteinase (half-filled diamonds), vs time of incubation. [phage] = 2.5×10^{11} phage particles/mL, [YO-PRO] = 10 μM, [BOXTO-PRO] = 9 μM, and [proteinase K] = 5×10^{-3} g/mL.

(8%), whereas the fraction of empty phages was somewhat higher in the BOXTO-PRO sample (13%). The 80% empty phages in the proteinase-treated sample is in good agreement with the amount of extracted DNA after 2 h of proteinase digestion, as measured by gel electrophoresis (Supporting Information).

Light Scattering. The effect of dye binding was also monitored by light scattering. Dynamic light scattering (DLS) provides information on the hydrodynamic properties of the phage particles, whereas the average scattered intensity reflects the amount of DNA inside the phage capsids.[10]

The nearly unaltered autocorrelation functions during the association of YO-PRO (Figure 10a) shows that the hydrodynamic properties of the phages are essentially unaffected by the dye binding and that they are similar to those of the native phages (open circles). By contrast, for the BOXTO-PRO sample (Figure 10b) the correlation function does change somewhat during the dye incubation, by deviating from native phages in the direction of longer correlation times. The diffusion coefficients derived from the short-time decay of the autocorrelation functions are shown in Table 3, which also presents the corresponding hydrodynamic radii. With the proteinase-treated sample (Figure 10c) the correlation function is seen to change consistently with digestion time giving two relaxation times.

The average scattered intensity during YO-PRO incubation (Figure 10d, squares) is essentially constant and equal to that of the native phages (which was constant for 24 h). By contrast, with BOXTO-PRO the intensity decreases with incubation time and finally reaches a level that corresponds to a 18% decrease compared to the intensity of native phages. For the proteinase-

TABLE 3: Diffusion Coefficients D_o and Hydrodynamic Radius R_h of the Phages (37 °C)

phage	D_o (μm^2/s)	R_h (nm)[d]
native phage	5.7	58
YO-PRO[b]	4.7	70
BOXTO-PRO[b]	2.4	140
proteinase K[c]	6.4	52

[a] After 3 h of incubation at 37 °C. [b] After 24 h of incubation at 37 °C. [c] After 6 h incubation at 56 °C. [d] Assuming spherical geometry.

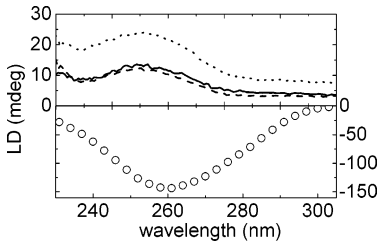

Figure 11. LD spectra of phage T5 in the native state (solid) and after incubation with YO-PRO (120 min; dashed), BOXTO-PRO (20 h; dotted), or proteinase (6 h; white circles). Note the different scales on the right and left axes, corresponding to negative and positive LD values, respectively. [DNA bp] = 13 μM, and [dye] = 9 μM.

treated sample the scattered intensity decreases steadily, as expected if the DNA is progressively released from the capsids, and reaches a plateau at approximately 15% of the level for the native phages.

This value is in good agreement with the 14% level which was observed after complete DNA ejection by use of the receptor FhuA.[10] The fraction f of DNA which is inside capsids will therefore be estimated from the fractional intensity I/I_o (Figure 10d) as

$$f = (I/I_o - 0.14)/(1 - 0.14) \quad (2)$$

Linear Polarized Spectroscopy. The linear dichroism of native phages is positive in the DNA absorption band around 260 nm (Figure 11, solid). By contrast, extracted T5 DNA (circles) exhibits a negative LD in this spectral region, as expected because free DNA polymer is oriented with its helix axis preferentially in the flow direction.[27] With YO-PRO and BOXTO-PRO bound to the phages (dashed and dotted, respectively), the positive LD is retained, which strongly indicates that the DNA is not released from the phages.

Discussion

Choice of Dyes. The set of asymmetric cyanine dyes in Figure 1 allowed the size, charge, DNA affinity, and binding mode to be varied in a systematic fashion. Furthermore, these dyes are strongly fluorescent in the bound state, while the fluorescence is essentially negligible when the dyes are free in aqueous solution.[19,21] For a given type of chromophore the fluorescence intensity will therefore be taken to be proportional to the bound amount of the corresponding dye molecules.

Status of Phage Particles. It is important to characterize the physical status of the phages after dye-binding, because the reduced fluorescence intensity compared to the extracted DNA (Figures 2 and 3c) could in principle arise because a corresponding fraction of the phages have been damaged and the dyes bind only to the released DNA. This possibility was investigated in detail with YO-PRO and BOXTO-PRO, because they represent different binding modes while otherwise being comparable regarding size and charge. The slow association compared to extracted DNA (Figure 2b,d) is not by itself

evidence of intact phages, because the phage particles may rupture only after the dyes have reached the interior of the capsid and perturbed the DNA inside for instance by extension. In previous studies the capsid status was monitored by ensemble-averaging methods, such as gel electrophoresis[16] or a DNAse protocol.[15] We choose to use electron microscopy as a complement to the ensemble methods of flow linear dichroism and dynamic and static light scattering.

Potential Release of DNA. The linear dichroism results (Figure 11) provide the most compelling support that the dye-incubated phages are intact. The LD of both YO-PRO and BOXTO-PRO stained phages is positive in the absorption band of DNA (centered at 260 nm), in agreement with the observation with the native phages. If the DNA was released from dye-treated phages due to capsid damage, it would be expected to exhibit a strong negative LD similar to the one observed with proteinase-digested phages.

Evaluation of the cryo-TEM images show that 8% of the native phages are empty (Table 2), which is in qualitative agreement with our fluorescence measurement that 2.5% of the total DNA in the native samples are present as free DNA. After YO-PRO staining the fraction of empty phages is unaltered (Table 2), which supports that the capsids are not ruptured when the dye binds to the phages. With BOXTO-PRO the fraction of empty phages is somewhat higher (13%); i.e., if anything BOXTO-PRO is worse than YO-PRO in damaging the phages. Similar conclusions are reached from the static light scattering results (Figure 10d). That the scattered intensity after YO-PRO staining is the same as with native phages strongly supports that the stained phages retain their DNA,[10] also after the 4 h incubation time which is required to reach equilibrium binding (Figure 2b). With BOXTO-PRO the scattered intensity has decreased by about 18% by the time (22 h) the association is complete (Figure 2d), suggesting that some DNA has been released from the phages.

In quantitative terms, we have to consider the possibility that about 20% of the phages have been ruptured and released their DNA, because it could explain the K_{rel} values we observe (0.18 for YO-PRO and 0.22 with BOXTO-PRO). With YO-PRO this possibility is all but excluded by the observation by cryo-TEM that the sample of stained phages contained no extra empty capsids (Table 2), in contrast to the 22% extra empty phages (i.e., 30% in total) which would be expected if ruptured phages was the explanation. Similarly, the scattered intensity after YO-PRO staining (Figure 10d) clearly does not exhibit the 33% decrease that would be needed, if the light scattering of empty phages is taken into account (see eq 2). Furthermore, if 22% of the phages indeed released their DNA, the flow LD amplitude (at 260 nm) should be about -30 in the units of Figure 11a, in contrast to the value $+10$ which is observed. Finally, if the fluorescence in the phage samples did arise because of free DNA from ruptured phages, these complexes should respond in the same way to changes in ionic strength as YO-PRO complexes with the extracted DNA. This is in disagreement with the observed effect of salt concentration on K_{eff} (Figure 7c).

The situation is less clear-cut with BOXTO-PRO. The electron micrographs do indicate a larger fraction of empty phages, but the 5% increase is considerably less than the 18% that would be expected if phage rupture was responsible. The 18% decrease in light-scattering intensity (Figure 10d), on the other hand, corresponds to a release of as much as 20% of the DNA after 22 h (see eq 2). This observation indicates that a substantial fraction of the phages have been damaged and released the DNA. However, this observation also raises the question why the excess amount of BOXTO-PRO we add to the phages does not lead to rupture of all phages. (The possibility that all phages have released 20% of their DNA is contradicted by the TEM pictures which show mostly empty or full phages and not that a majority of the phages are partially empty.) The LD results support that the phages are intact, because the amplitude should be about -13, whereas a value of $+20$ is found (Figure 11).

Phage-Aggregation with Dyes. The tendency of the phages to aggregate after staining with YO-PRO (Figure 9c) is not observed with native phages, indicating that it is an effect of the dye. We have not studied this dye-induced aggregation in detail, but the approximately circular shape with the capsids at the periphery indicates that the tails are involved. One possibility is that the tails are negatively charged as with T7[28] and bridged by the divalent cationic dyes, which are in excess. Nonelectrostatic interactions may also be involved, however, because the tendency of phage aggregation seems to be stronger with BOXTO-PRO which is also divalent but expected to be less hydrophilic.[21] It should be remembered, however, that the two samples were exposed to dyes for different times.

The dynamic light scattering measurements give a hydrodynamic radius of $R_h = 58$ nm for native T5 phages (Table 3), in agreement with earlier studies.[10] After YO-PRO or BOXTO-PRO association, the apparent phage size is larger (Table 3) but not by the factor of $5-10$ that would be estimated if the phages were aggregated to the extent seen by cryo-TEM (Figure 9c,d). For the moment we cannot exclude that the aggregates are an effect of the sample preparation for electron microscopy, although the R_h values suggest that dye-stained phages do not diffuse as independent particles.

Analysis of the Proteinase-Treated Phages. The DLS results after dye staining (Table 3) appear to support that the phages are intact, because R_h is not smaller (than for native phages) as otherwise may have been expected if the phages became fragmented. Interpretation of the DLS results in terms of DNA release requires some caution, however, in view of the results on the proteinase-treated sample (2 h), which was included to have a control where a fraction of the phages had been damaged by intention. The cryo-TEM images (Figure 9b) indicate that capsids do not have to be visibly fragmented in order to release the DNA, since empty capsids often retain the characteristic shape of native phages. This observation suggests that phage DNA can escape through holes in the capsid which are too small to be detected by electron microscopy. Furthermore, the essentially retained capsid geometry implies that the effective hydrodynamic radius of such empty phages cannot be expected to differ significantly from that of native phages.

The cryo-TEM images of the proteinase control also show both partially emptied phages (Figure 9b) and fragmented capsids (results not shown), albeit the weak contrast of individual DNA strands[29] prevented us from locating the released DNA. The combined cryo-TEM observations of DNA release and capsid fragmentation should be compared to the DLS results on proteinase-treated phages (Figure 10c). Scattering from free DNA is negligible even if all the phage DNA has been released,[10] so the scattering intensity is expected to be governed by the capsids and capsid fragments. The DNA may still affect the correlation curves indirectly. When the capsids are only partially empty, the released DNA is expected to still be attached to the phage (the T5 genome is a single molecule) and may confer a retarding hydrodynamic drag on the phage. One possibility is that the shift to longer correlation times at low degrees of correlation reflects this enhanced drag of "hairy"

phages, whereas the oppositely directed shift at high degrees of correlation reflects the faster diffusion of capsid fragments (compared to intact phages).

Affinity of Dyes to the T5 Phage. We use the ratio K_{rel} (Figure 7c) as a measure for the degree of dye binding to the T5 DNA when it is inside the capsid compared to when it is free in solution under otherwise identical conditions. In this approach, the 6 h proteinase-treated sample ("extracted DNA") should be good representative for free T5 DNA, since the results from static light scattering (Figure 10d) and gel electrophoresis (Supporting Information) agree that the DNA is fully released from the capsid.

We find that K_{rel} is smaller than unity for all dyes studied here (Figure 7c); i.e., the binding is weaker when the T5 DNA is inside the phage capsid, compared to when the DNA is free in solution. This fluorescence-based observation is supported by the properties of the absorption spectra, as pointed out in connection with Figure 5. Such a reduction in dye-binding when the DNA is packed inside the phage capsid seems to be a general effect. Two earlier reports[15,16] on two distinctly different dye−phage systems showed that the affinity constant is reduced, whereas the number of binding sites is the same as when the DNA is free in solution. We will therefore discuss K_{rel} in terms of effects on the affinity constant, but it should be remembered that it is still an assumption in the T5 case that the number of binding sites is the same for free and capsid-confined DNA.

Comparison of Dyes. For intercalating dyes one possible reason for the decrease in relative affinity[15] is that the required helix extension and unwinding is energetically unfavorable since the DNA is already densely packed inside the capsid. However, this cannot be the only reason for the reduced dye affinity for phages, since K_{rel} is well below unity also for the groove-binding BOXTO-PRO (Figure 7c). In fact, its relative affinity is even lower than for the closely related intercalator YO-PRO (Figure 3c), which indicates that the strength of the nonextension type of penalty for groove binders is comparable to that caused by helix perturbation. A possible second explanation for the reduced phage affinity[15] is competition with multivalent cations that occur naturally in some phages.[30] Such agents, for instance spermine and spermidine, are sometimes groove-bound. BOXTO-PRO is then expected to be particularly exposed to competition since it shares their binding mode, which may explain the particularly low value of K_{rel} for BOXTO-PRO (Figure 7c). Added spermine is known to enter the T5 capsid and reduce the driving for DNA ejection,[10] but the role of polyamines in the native T5 has to our knowledge not been ascertained.[30]

The above interpretations of K_{rel} rely on the assumption that the binding modes of YO-PRO[17] and BOXTO-PRO,[21] which were determined with DNA free in solution, also apply when the DNA is inside the T5 capsid. The spectral similarity in both emission (Figure 4) and excitation spectra (Supporting Information) strongly supports that YO-PRO binds in the same manner to DNA inside phages as to DNA free in solution (i.e., by intercalation). With BOXTO-PRO these types of spectra do differ somewhat between phages and extracted T5 DNA (Figure 4), in particular in the initial stage of the association, but less so when binding is complete. Notably the initial red shift resembles the effect seen in the (weak) fluorescence from BOXTO-PRO free in water.[21] One possibility is that the BOXTO-PRO fluorescence initially reflects association to an environment (for example, the outside of the helix) which is more polar than the final binding site in the groove. However, for the sake of our argument, it is sufficient to observe that it is unlikely that BOXTO-PRO intercalates when the DNA is inside the phage. Comparison of a large set of cyanine dyes[31] indicates that groove-binding of dyes such as BOXTO-PRO is due to that it is crescent-shaped and long enough, dye properties which are expected to remain when the dye is inside the capsid. The detailed mode of BOXTO-PRO-binding to DNA inside the capsid is presently being investigated by LD measurements on dye-stained phages, which can be oriented by flow (Figure 11). The potential of this approach is demonstrated by the conclusion that helical axis is preferentially oriented perpendicular to the direction defined by the tail (which most likely determines the direction of flow alignment), which can be drawn from the positive LD for the native phages.

Intercalators may also suffer from competition from capsid-intrisic cations, at least electrostatically. It is therefore noteworthy that, within the set of investigated intercalators (Figure 1), it is YOYO which suffers most in phage affinity (relative to extracted DNA), even though YO-PRO and YO have lower charge and therefore should be less efficient in electrostatic competition with intrinsic cations. In fact, YO-PRO and YO have similar K_{rel} values (Figure 7c) which suggests that neither the extra charge nor the tail of YO-PRO contributes strongly to the reduced phage affinity of the YO family of dyes. The extra strong penalty for YOYO binding to DNA inside the phage (Figure 7c) therefore seems to be related to its dimeric nature. The helix perturbation by one YOYO is more localized than with two YO-PRO and may therefore be energetically costlier to accommodate when the T5 DNA is densely packed inside the phage than when free in solution. Notably, these observations are based on K_{rel} data at 150 mM NaCl, but as discussed below, the situation may be different at other ionic strengths.

Effect of Ionic Strength. The results of Figure 7 show that the YO and YO-PRO affinity to the phages is screened by added salt (Figure 7a) in a manner similar to extracted DNA (Figure 7b). This observation supports that the dye-binding to phage is indeed to its DNA. Furthermore, it indicates that the ionic environment inside the T5 capsid can be affected by the external ionic strength, in agreement with that externally added Cu^{2+} ions quench the fluorescence of EB bound to phage T7.[13]

To separate electrostatic and nonelectrostatic contributions to the affinity, we exploit the observation[32] that the ionic strength of 1 M NaCl is high enough to fully screen electrostatic interactions in DNA systems. This proposition is supported in the present DNA−dye system by the observation that YO-PRO exhibits higher DNA affinity (intensity) than YO (Figure 7b), except at 1 M NaCl where it is the same. If we turn to phage-binding, it is noteworthy that K_{rel} is higher for YO than for YO-PRO at 1 M NaCl (Figure 7c), which thus indicates that the PRO tail in fact confers a nonelectrostatic penalty for binding to the DNA inside the phage (compared to DNA free in solution). This observation suggests that there is a steric hindrance to accommodate the tail in the densely packed DNA.

The decrease in K_{rel} when the ionic strength is reduced below 1 M NaCl (Figure 7c) indicates a nonfavorable electrostatic effect on dye-binding to DNA inside the phages. This observation may seem unexpected because electrostatic interactions usually favor binding of cationic dyes to DNA, as seen from the increasing intensity with decreasing ionic strength in Figure 7b. However, K_{rel} reflects the differential dye binding compared to the same phage DNA when free in solution. The decrease in K_{rel} in Figure 7c thus indicates that the electrostatic attraction is comparatively weaker for binding to the phage DNA when it is inside the capsid, at a given external ionic strength. This observation suggests that the intracapsid environment has a higher effective ionic strength than the external solution, possibly

due to a generally higher concentration of simple salts or the presence of multivalent DNA-binding molecules. We conclude that the ionic strength inside the T5 capsid is in a sense buffered, at least when probed by cationic DNA-binding dyes.

Association Kinetics. There are at least four possible processes that may contribute to the kinetics of dye association to a bacteriophage:[13,14]

(I) binding of the dye to the capsid surface (which may give a spectroscopic response in our system since cyanine dyes are expected to exhibit some degree of fluorescence enhancement upon immobilization to any surface);

(II) dye penetration through the capsid shell and binding to the closest DNA layer(s) inside it;

(III) dye diffusion through the remaining part of the densely packed DNA matrix inside the capsid;

(IV) binding to the actual site on the individual DNA helices. This process may be retarded if the DNA is densely packed, potentially to the extent that process IV is slower than process III.

As opposed to earlier studies[13,14,16] we choose to use a condition of excess dye, to suppress back-reactions in the processes above. Experimentally we observe two rates (k_1 and k_2) that are associated with dye-binding to the phages. In addition there is a very fast initial step, I_o, in eq 1, which we assign to dye-binding to the free DNA which is present in our phage preparation. This assumption is reasonable since a correspondingly small fraction of the phages are empty according to cryo-TEM (Table 3), but we cannot rule out that the initial step has a contribution from process I because the estimated diffusion-controlled association time under our conditions is about 0.1 s and, therefore, similar to that for free DNA. The latter estimate also means that the two processes we observe (k_1 and k_2) are too slow to correspond to process I. Process IV seems unlikely to be rate-limiting in our system, since both k_1 and k_2 are lower for BOXTO-PRO than for YO-PRO (Figure 3a). It is difficult to envisage a mechanism where dense DNA packing retards helix-binding more for a groove-binding dye than for an intercalator.

Serwer and co-workers[13] reported that shell penetration (process II) is not rate limiting for EB association to T7, because mutants with different amounts of DNA but the same capsid shell exhibited different rates of association. The properties of the T5 and T7 phages may be quite different, though, as indicated by the quite different association rates for YO and EB (which are comparable in size and charge). EB needs about 15 min to fully bind to phage T7 at 37 °C, whereas YO associates to T5 in 2−3 min. In fact, dye association to T5 phages seems unusually fast, because the monovalent and similarly sized PF needs about 7 h to fully associate to phage T2,[14] whereas EB does not enter at all.[33]

We therefore propose that the two experimental rates we observe kinetically reflect processes II and III, which notably are sequential in nature because the dye cannot diffuse through the DNA matrix inside the capsid until it has penetrated its shell. Here we test the proposed kinetic scheme against the available data, although a full verification clearly needs additional experiments.

First we note that k_1 (at 150 mM NaCl; Figure 3a) decreases with increasing effective size of the dyes, which is assumed to increase in the order YO, YO-PRO, BOXTO-PRO, and YOYO (Figure 1). (For YO we estimate a lower limit of 0.01 s^{-1} for k_2 (and hence k_1) from Figure 2a). This order of k values is consistent with the permeation being governed by steric constraints due to an effective pore size of the capsid shell. In addition we find that k_1 (for YO-PRO) increases with increasing ionic strength (Figure 8a), which suggests that the effective pore size includes an electrostatic contribution. In fact, irrespective of the effective sign of the capsid charge in the pore (whether repulsively cationic or attractively anionic), screening of the electrostatic force between the dye and the capsid proteins is expected to facilitate the penetration of the dyes. We cannot exclude, however, that the capsid itself is affected by the ionic strength, for instance by screening of electrostatic interactions between the proteins that make up the shell.

Second, if we are correct in the conclusion that dye diffusion (process III) is slower than dye association to the helix (IV) (see above), each dye will associate and dissociate many times during the spreading throughout the capsid interior. The effective rate of dye diffusion is then expected to be governed by the fraction of time the dye is nonbound (i.e., be inversely related to its affinity constant for DNA) and by the rate of transport of (nonbound) dye through the DNA maze (i.e., primarily by the effective dye size). These predictions are also in accord with observation, since k_2 decreases in the same order YO, YO-PRO, BOXTO-PRO, and YOYO as the estimated size and affinity constants do. Figure 8a indicates that the effect of salt concentration on k_2 is relatively weak. This observation is in apparent contrast to the proposed role of the affinity constant in process III: intrashell diffusion of a cationic dye should in effect be faster with increasing ionic strength since the lifetime of its unbound state increases when the affinity to DNA decreases. However, as noted above, electrostatic effects inside the capsid seems to be buffered to a certain extent by the intracapsid ionic environment. Finally, the relative amplitudes of the two experimental processes vary with ionic strength (Figure 8b). This observation indicates that the amount of DNA which is involved in the two steps varies with ionic strength. One possibility is that the number of DNA layers which becomes dye bound directly after penetration increases with increasing ionic strength, because dye diffusion between DNA layers is faster. This hypothesis is supported by the fact that A_1 (at 150 mM NaCl) is higher the smaller the dye (Figure 3b); i.e., the slower the expected diffusion between DNA layers (now because of dye size) the smaller the value of A_1.

A final point of discussion should regard potential Donnan effects. The high permeability of the capsid shell to YO suggests that ion and water equilibria will be established quickly (minutes or faster). Osmotic effects seem small, since our results show that capsids are not ruptured even at high ionic strength. The Donnan potential that would arise can be estimated to be as high as 100 mV (negative inside), using a simple ideal-solution approach. This potential may contribute an electrophoretic component to the penetration of the cationic dyes.

Conclusions

Unsymmetrical cyanine dyes are useful as spectroscopic probes for bacteriophage T5, due to their excellent fluorescent properties. We found that intercalated dyes are advantageous as spectroscopic probes with T5, because YO-PRO-binding does not damage the capsids, whereas there is a possibility that groove-binding BOXTO-PRO does. The very fast association with YO indicate that the capsid-wall permeability is unusually high for T5, compared to the permeability of both T7 and some T-even phages (T2, T4), as investigated with probes of same charge and comparable size.

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Supporting Information Available: Details of the gel electrophoretic analysis of the proteinase-extracted bacteriophage T5 DNA, numerical values of the fitted kinetic constants for the dye association to bacteriophage T5, and excitation spectra of YO-PRO and BOXTO-PRO bound to phage T5. This material is available free of charge via the Internet at http://pubs.acs.org.

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