Exchange of Counterions in DNA Condensation

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Abstract

We measured the fluorescence intensity of DNA-bound fluorescent dyes YO-PRO-1 (oxazole yellow) and YOYO-1 (dimer of oxazole yellow) at various spermidine concentrations to determine how counterions on DNA are exchanged in the process of DNA condensation. A decrease of fluorescence intensity was observed with an increase of spermidine. Considering the chemical equilibrium under the competition between the dye and spermidine for counterion condensation on DNA, the theoretical curve well describes the decrease of the fluorescence intensity. These results indicate that dyes are exchanged for spermidine at the binding site on DNA; that is, the exchange of counterions occurs. The parameters associated with the decrease of the fluorescence intensity show that the relative affinity of the dye and spermidine for DNA depends on the state of DNA. Moreover, YOYO-1 prevents the DNA condensation, but the effect of YO-PRO-1 on the condensation is very slight, though both dyes intercalate for DNA; the high affinity of YOYO-1 compared to YO-PRO-1 enables prevention of the condensation.
INTRODUCTION

A DNA molecule is negatively charged and takes on the form of a disordered coil in aqueous solution containing monovalent counterions. When multivalent cations such as spermidine (3+) and spermine (4+) are in the solution, however, the DNA molecule collapses or condenses and forms a tightly packed structure above a certain concentration of multivalent cations\(^1\). This phenomenon is well known as DNA condensation, but it cannot be understood within the context of mean-field theories such as the Poisson-Boltzmann theory which always gives a repulsion between polyelectrolytes regardless of the valence of any added salt\(^2\). Like-charge attractions have been widely observed in biopolymer systems such as DNA, actin, and microtubule, which is attracting much interest in physics, chemistry, and other fields\(^3\); the packaging of DNA in viruses, cells, and liposomes using this phenomenon may contribute to gene delivery\(^4\). In DNA condensation, the behavior of counterions in solution plays a key role. According to counterion condensation theory\(^5-7\), counterions are condensed on DNA and screen the DNA charge. The neutralization ratio \(\psi\) for duplex DNA depends on the valence \(m\) of the counterion; \(\psi = 0.76, 0.88, \) and \(0.92\) for \(m = 1, 2, \) and \(3,\) respectively. As multivalent cations are added, counterions screening DNA are exchanged for multivalent counterions. For the DNA condensation, about 90% of charge neutralization for DNA is indispensable\(^1\).

In this study, to determine how counterions on DNA are exchanged due to adding multivalent cations, we measured the fluorescence intensity of DNA-bound YO-PRO-1 or YOYO-1 at various spermidine concentrations. Because of their high affinity, high fluorescence intensity and low background\(^8,9\), these dyes are often used as fluorescent probes in DNA analysis. YOYO-1 (4+) has double the charge of YO-PRO-1 (2+), and they are bis- and monointercalator, respectively\(^10\). Both dye and spermidine have positive charges, and they interact with DNA as counterions. Thus, if dyes binding to DNA are exchanged for added spermidine, this exchange can be observed through the change of the fluorescence intensity. Recently, the decrease in the fluorescence intensity of DNA-bound YOYO-1 during DNA condensation has been observed\(^11\). The fluorescence intensity shows a dramatic decrease in the condensation by the multivalent cation \(\text{Co(NH}_3)_3\text{H}_2\text{O}^{3+}\). For both YO-PRO-1 and YOYO-1, we also observed the decrease of the fluorescence intensity with the increase of spermidine. The theoretical curves well fitted the measurements, which indicates that dyes are exchanged for spermidine,
namely, the exchange of counterions on DNA occurs. The parameters associated with the decrease of the fluorescence intensity were obtained by fits, which shows that the affinity of the dye depends on the state of DNA. The single molecule observation using fluorescence microscopy shows that the intercalator, YOYO-1, prevents the DNA condensation, whereas the effect of the minor-groove binding dye, DAPI, on the condensation is negligible\textsuperscript{12}. In our experiments, the effect of YO-PRO-1 to prevent the DNA condensation was much less than that of YOYO-1, although both dyes are intercalators. It is assumed that the high affinity of YOYO-1 compared to YO-PRO-1 is the reason that condensation is prevented.

**MATERIALS AND METHODS**

Lambda phage DNA (Promega, USA) was stocked in buffer (10 mM Tris-HCl, pH 7.0) with the base pair (bp) concentration of 10 $\mu$M. YO-PRO-1 and YOYO-1 (Molecular Probes, USA) were diluted in buffer with a concentration of 10 $\mu$M before use. Spermidine trihydrochloride (Sigma, USA) was dissolved in buffer and stocked with concentrations of 1 mM and 10 mM. All solutions used Millipore water (18.2 M$\Omega$). First, DNA and the dye were mixed in buffer and incubated at room temperature for 1 h. Then an appropriate amount of spermidine solution was added and incubated for at least 1 h. The mixed solutions were protected from light during the incubation to reduce the fading of the fluorescence. After the incubation, one half of the solution was used for the fluorescence intensity measurements as it was, and the other half of it was used for precipitation experiments. In all experiments, the ratio of the concentration of the dye and DNA (bp) was 1:2. The final concentrations of the dye and DNA (bp) were 0.05 and 0.1 $\mu$M, respectively.

The fluorescence intensity of solutions was obtained using a fluorescence spectrophotometer (HITACHI, F-2500) at 24±1°C. For both dyes, the excitation wavelength was 491 nm, and the emission was 509 nm with slit widths set at 5 nm. To estimate the critical concentration of spermidine for DNA condensation to occur, precipitation experiments\textsuperscript{13,14} were performed. One half of the solution after the incubation was centrifuged at 10,000 rpm for 10 min at room temperature. Then the supernatant was put into another tube, and the fluorescence intensity of the supernatant was measured. When the spermidine concentration reaches the critical concentration for DNA condensation, DNAs are collapsed or condensed and most DNAs precipitate at the bottom of the tube. At each spermidine concentration,
the ratio of the fluorescence intensity $I_{sup}/I_{sol}$ was measured. Here, $I_{sup}$ is the fluorescence intensity of the supernatant and $I_{sol}$ is the intensity of the solution not centrifuged. The dependence of $I_{sup}/I_{sol}$ on spermidine concentration indicates the occurrence of DNA condensation.

RESULTS AND DISCUSSION

Fluorescence intensity measurements

The fluorescence intensity and the ratio, $I_{sup}/I_{sol}$, at various spermidine concentrations are shown in Fig. 1. We subtracted the fluorescence intensity of the buffer from the measurements, which was about $10^2$ times lower than that in the presence of the dye and DNA. The fluorescence intensity of each dye in the absence of DNA was several orders of magnitude lower than that of the dye-DNA complex. The spermidine concentration, $x$, varied from 0 to 0.2 mM for YO-PRO-1, and from 0 to 3 mM for YOYO-1. The fluorescence intensity at each spermidine concentration was normalized by the intensity at $x = 0$. For both dyes, the fluorescence intensity decreased as the spermidine concentration increased. This suggests that the dyes are detached from DNA, and the DNA is screened by spermidine instead, namely, the exchange of counterions occurs. Though both intensities, $I_{sup}$ and $I_{sol}$, decreased due to the dissociation of dyes, the $I_{sup}/I_{sol}$ decreased above the spermidine concentration of 0.13 mM for YO-PRO-1 and 0.5 mM for YOYO-1. The results of the precipitation experiments show that the DNA condensation occurs at a spermidine concentration above 0.13 mM for YO-PRO-1 and 0.5 mM for YOYO-1.

Exchange of dyes for spermidine

To elucidate the exchange of dyes for spermidine at the binding sites on DNA, we consider the chemical equilibrium under the coexistence of dyes and spermidine. When both dye ($F$) and spermidine ($S$) are present in the solution, competition occurs between the dye and spermidine for the binding sites ($D$) on DNA.
When the system is in equilibrium,

\[
D + F \xrightleftharpoons{K_F} DF, \quad K_F = \frac{[DF]}{[D][F]},
\]

\[
D + nS \xrightleftharpoons{K_S} DS_n, \quad K_S = \frac{[DS_n]}{[D][S]^n},
\]

\[
DS_n + F \xrightleftharpoons{\alpha} DF + nS, \quad \alpha = \frac{[DF][S]^n}{[DS_n][F]} = \frac{K_F}{K_S},
\]

where \(DF\) and \(DS_n\) represent the dye-DNA and the spermidine-DNA complex, respectively; \(n\) represents the number of spermidine that are condensed and screens a binding site on DNA instead of a dye; \(K_F\) and \(K_S\) represent the equilibrium constant of the dye and spermidine, respectively. In fact, the dye binds to the binding site and spermidine only screens the DNA; however, we regard the screened DNA as a complex of DNA and spermidine for convenience. The conservation of mass gives

\[
[D] + [DF] + [DS_n] = D_{tot}^{site},
\]

\[
[F] + [DF] = F^{tot},
\]

\[
[S] + n[DS_n] = x,
\]

where \(D_{tot}^{site}\), \(F^{tot}\), and \(x\) represent the total concentration of the binding site, the dye, and spermidine in the solution, respectively. Here, we neglect \([D]\) in Eq. (4) and \(n[DS_n]\) in Eq. (6) for the following reasons: 1) the dyes bind so strongly to DNA (\(K_F = 10^5\)-\(10^7\) M\(^{-1}\) for YO-PRO-1\(^{16}\) and \(10^{10}\)-\(10^{12}\) M\(^{-1}\) for YOYO-1\(^{8}\)) that most of the binding sites are occupied by dyes or spermidine; 2) the spermidine concentration in the solution is about \(10^2\)-\(10^4\) times larger than the concentration of the binding site. Solving the simultaneous equations, Eq. (3)-(6), and assuming that the fluorescence intensity \(I\) is proportional to \([DF]\), we obtain

\[
\frac{I(x)}{I_0} = C^{-1}[DF] = \frac{\alpha(D_{tot}^{site} + F^{tot}) + x^n - \sqrt{\alpha^2(D_{tot}^{site} - F^{tot})^2 + 2\alpha(D_{tot}^{site} + F^{tot})x^n + x^{2n}}}{2\alpha C},
\]

\[
C = \begin{cases} 
F^{tot} & \text{if } D_{tot}^{site} \geq F^{tot} \\
D_{tot}^{site} & \text{if } D_{tot}^{site} < F^{tot}
\end{cases},
\]

where \(I_0\) is the intensity at \(x = 0\). The simultaneous equations have two positive solutions for \([DF]\); however, only the smaller solution satisfies the constraints in Eq. (4) and (5) when
$x$ increases. Here, $D_{\text{site}}^{\text{tot}}$ is still unknown, because the number of base pairs per binding sites is still unknown though we know the total base pair concentration $D_{\text{bp}}^{\text{tot}}$ ($=0.1$ μM). Then we assume that DNA is neutralized with the ratio $\psi$, which is determined using counterion condensation theory.$^{5-7}$ The $\psi$ means the average number of the positive charge for a negative charge of DNA; $\psi = 0.92$ for spermidine ($m = 3$). We rewrite Eq.(3) with the number of charges as

$$F^{z+} + DS_n \rightleftharpoons DF + nS^{m+},$$

(9)

$$z + (\psi q - q) = (z - q) + nm,$$

(10)

where $q$ represents the number of charges of DNA in a binding site, and $z$ represents the valence of the dye molecule; $z = 2$ and 4 for YO-PRO-1 and YOYO-1, respectively. Equation (10) means the charge balance, which leads to $q = nm/\psi$. Thus, the total concentration of the binding site is given by

$$D_{\text{site}}^{\text{tot}} = \frac{D_{\text{bp}}^{\text{tot}}}{N_b} = \frac{0.613D_{\text{bp}}^{\text{tot}}}{n} \quad \text{(for } m = 3),$$

(11)

where $N_b = q/2$ is the number of bp per site because DNA has two negative charges per bp. Substituting Eq. (11) into Eq. (7), the fluorescence intensity is described as a function of $x$ with parameters, $n$ and $\alpha$. We analyzed the measurements in experiments using Eq. (7) and obtained $n$ and $\alpha$ by the multiparameter fits using the Levenberg-Marquardt algorithm.

Figure 2 shows the dependence of the fluorescence intensity in Fig. 1 with theoretical curves in Eq. (7)$^{15}$. For both dyes, the decrease of the fluorescence intensity changed at a certain concentration $x_c$ of spermidine and it was difficult to fit using a single curve in the entire range of spermidine concentrations; $x_c = 0.14$ mM for YO-PRO-1 and 1.2 mM for YOYO-1. The insets in Fig. 2 clearly show that the fluorescence intensity for $x > x_c$ decreases faster than that for $x < x_c$. Therefore, the fits were performed within each range, $x < x_c$ and $x > x_c$. We substituted $x - x_c$ for $x$ in Eq. (7) in the range $x > x_c$, and the optimal fits were obtained by the above values of $x_c$. The theoretical curves well fit the measurements within each range, which shows that the exchange of dyes for spermidine at the binding site causes the decrease of the fluorescence intensity.

The results of the precipitation experiments in Fig. 1 show that $I_{\text{sup}}/I_{\text{sol}}$ decreases above the spermidine concentration of 0.13 mM for YO-PRO-1 and 0.5 mM for YOYO-1. It suggests that the DNA condensation occurs above these values, and for $x \geq x_c$, DNA
molecules are in the collapsed state for both dyes. Thus, the different decrease of the fluorescence intensity at below and above $x_c$ is due to the different states of DNA molecules. In the case of YO-PRO-1, the value of $x_c$ is almost the same value where DNA condensation occurs in the absence of dyes. The $x_c$ for YOYO-1 is several times larger than that for YO-PRO-1. The previous study shows$^{12}$ that the intercalator, YOYO-1, prevents the DNA condensation, whereas the effect of the minor-groove binding dye, DAPI, on the condensation is negligible. Our results indicate that YOYO-1 prevents DNA condensation but the effect of YO-PRO-1 on the condensation is very slight, though both dyes are intercalators.

**Parameters obtained by fits**

The parameters, $\alpha$ and $n$, obtained by the fits are shown in Table I. The ratio $\alpha(= K_F/K_S)$ for $x > x_c$ was about 2.8 and 4.0 times smaller than that for $x < x_c$ in the case of YO-PRO-1 and YOYO-1, respectively. The decrease of the fluorescence intensity becomes more rapid for small $\alpha$ compared to large $\alpha$ because the dye binding on DNA can easily be exchanged for spermidine when $\alpha$ is small. The affinity of dyes for DNA depends on the salt concentration in the solution, which decreases as the salt concentration increases$^{16}$. Considering only the dye-DNA reaction in Eq. (1), $K_F$ depends on the spermidine concentration and could decrease with the increase of the spermidine concentration, since the binding of spermidine to DNA leads to the decrease of $[DF]$. The ratio $\alpha$, however, means the relative affinity of the dye and spermidine for DNA. If the chemical equilibrium in Eq. (3) is established, no change of $\alpha$ would be observed for all spermidine concentrations. Thus, the change of $\alpha$ at below and above $x_c$ indicates that the relative affinity depends on the state of DNA; the relative affinity for the collapsed DNA is smaller than that for the elongated DNA. Moreover, the $\alpha$ for YOYO-1 was about 100 times larger than that for YO-PRO-1, which shows that YOYO-1 prevents the DNA condensation due to its high affinity for DNA, but the effect of YO-PRO-1 on the condensation is very slight. It is assumed that the change of the relative affinity is mainly due to the change of the dye’s affinity for DNA. When DNA is in a collapsed state, the collapsed DNA prefers to form a toroidal structure whose diameter is about 100 nm$^{17,18}$. To form a toroidal structure, DNA must be bent against its persistence length of about 50 nm. The average molecular weight for DNA is about 660/bp, and it is comparable with that for each dye molecule and several times larger.
than spermidine; the molecular weight is 375.5 for YO-PRO-1(2+), 763.1 for YOYO-1(4+), and 148.2 for spermidine(3+). Therefore, the deformation of DNA has a more significant effect on a dye’s affinity than the affinity of spermidine. The large decrease of $\alpha$ for YOYO-1 compared to YO-PRO-1 supports the above conjecture.

For $x < x_c$, it is reasonable that the $N_b$ of YOYO-1 is larger than that of YO-PRO-1, because the former is a dimer of the latter. The $N_b$ for YO-PRO-1 is consistent with the value for the solution containing 20 - 100 mM sodium ions\textsuperscript{16}. If the mode of binding is monointercalation for YO-PRO-1 and bisintercalation for YOYO-1, the latter would need more than double the $N_b$ of the former. The $N_b$ obtained for YOYO-1, however, was about 1.3 times larger than that of YO-PRO-1. There are two binding modes for both dyes binding to DNA; the first mode is intercalation and the second is external binding, which depends on the mixing ratio between dye and DNA (bp)\textsuperscript{10}. At a mixing ratio up to 0.1 for YO-PRO-1 and 0.0625 for YOYO-1, the predominant mode is intercalation, and at the ratio above these values external binding to DNA occurs. In our case, the external binding to DNA occurred for both dyes, because the mixing ratio was 0.5. The $N_b$ obtained by the fits is average for all binding sites of DNA. In fact, $N_b$ at each binding site would vary depending on the binding mode of the dye. If the difference of $N_b$ between two dyes with external binding mode is much less than that with the intercalation, the small difference in $N_b$ between the two dyes can be explained. For $x > x_c$, the $N_b$ was larger than that for $x < x_c$ in the YO-PRO-1 case, while in the YOYO-1 case the $N_b$ was almost the same for each range. Here, it should be emphasized that $n$ is the number of spermidine screening DNA instead of a dye, and $N_b$ was led by $n$. For YOYO-1, the result shows that the $n$ is almost independent of the state of DNA, though its affinity depends on the state. The high affinity of YOYO-1 might cause the difference in the change of $N_b$ between two dyes.

**Change of neutralization ratio**

If all binding sites are occupied by dyes at $x = 0$, the normalized fluorescence intensity means the ratio of binding sites occupied by dyes to all sites. The neutralization ratio of the binding site for dye-DNA is different from spermidine-DNA. We simply estimated the neutralization ratio of DNA-bound dye and spermidine. We define the neutralization ratio
TABLE I: Parameters obtained by the fits. $N_b$ was led using $n$ obtained by the fits.

<table>
<thead>
<tr>
<th></th>
<th>$x &lt; x_c$</th>
<th>$x &gt; x_c$</th>
<th></th>
<th>$x &lt; x_c$</th>
<th>$x &gt; x_c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$</td>
<td>$2.2 \pm 0.1 \times 10^2$</td>
<td>$0.8 \pm 0.4 \times 10^2$</td>
<td>$\alpha$</td>
<td>$1.7 \pm 0.1 \times 10^4$</td>
<td>$0.43 \pm 0.02 \times 10^4$</td>
</tr>
<tr>
<td>$n$ (1/site)</td>
<td>$1.2 \pm 0.1$</td>
<td>$1.8 \pm 0.1$</td>
<td>$n$ (1/site)</td>
<td>$1.6 \pm 0.1$</td>
<td>$1.4 \pm 0.1$</td>
</tr>
<tr>
<td>$N_b$ (bp/site)</td>
<td>$2.0 \pm 0.2$</td>
<td>$2.9 \pm 0.2$</td>
<td>$N_b$ (bp/site)</td>
<td>$2.6 \pm 0.2$</td>
<td>$2.3 \pm 0.2$</td>
</tr>
</tbody>
</table>

\( \Phi \) as an average for all sites occupied by dye or spermidine, and then \( \Phi \) is described as

\[
\Phi = I \times \frac{z}{q} + (1 - I) \times \psi ,
\]  

(12)

where \( I \) is the fluorescence intensity, \( z/q (q = 2N_b) \) and \( \psi (= 0.92) \) represent the neutralization ratio of DNA-bound dye and spermidine, respectively. The decrease of the fluorescence intensity changes at \( x = x_c \) for both dyes and it is assumed that DNAs are collapsed in \( x > x_c \). Using Eq. (12), \( N_b \) for \( x < x_c \) in Table I, and the intensity at \( x_c = 0.14 \) mM for YO-PRO-1 and 1.2 mM for YOYO-1, the neutralization ratio \( \Phi^* = 0.88 \) was obtained for both dyes. The neutralization ratio estimated here is rather small compared to the value of 0.89-0.90 given in the previous study\(^1\). In our estimation, we ignored the effect of monovalent counterions for simplicity. In fact, the ratio is supposed to be a little larger than the value estimated here. Since the buffer solution includes Tris\(^+\) ions, taking the effect of the monovalent counterions into consideration, another 0.01-0.02 neutralization ratio could be achieved. Although this effect should be considered carefully, the coincidence of \( \Phi^* \) for both dyes in our estimation suggests that the DNA condensation occurs when \( \Phi \) reaches \( \Phi^* \) at \( x = x_c \). A schematic diagram of this behavior is shown in Fig. 3.

CONCLUSIONS

The exchange of counterions in DNA condensation was observed as the decrease of the fluorescence intensity of DNA-bound fluorescent dyes YO-PRO-1 and YOYO-1. The theoretical curve considering the chemical equilibrium under the competition between the dye and spermidine well describes the measurements obtained in experiments. The ratio \( \alpha \), which means relative affinity of the dye and spermidine for DNA, was obtained by the fits...
for measurements. For both dyes, the ratio $\alpha$ for a collapsed DNA was several times smaller than that of an elongated DNA, which suggests that the affinity of the dye for DNA depends on the state of DNA; the affinity for a collapsed DNA is smaller than that for an elongated DNA. For this reason, the decrease of the fluorescence intensity becomes more rapid for collapsed DNA compared to elongated DNA. YOYO-1 prevents the DNA condensation, but the effect of YO-PRO-1 on the condensation is very slight, though both dyes intercalate for DNA; the high affinity of YOYO-1 compared to YO-PRO-1 enables prevention of the condensation.

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To fit the measurements, $C$ in Eq. (7) must be specified. The fits were performed in each case in Eq. (8) and except for $x < x_c$ in YO-PRO-1, the curves clearly well fitted measurements in $C = D_{\text{tot site}}^{\text{tot}}$. For $x < x_c$ in YO-PRO-1, the difference between the two cases was slight because both results showed $D_{\text{site}}^{\text{tot}} \sim F^{\text{tot}}$; the solid line shown in Fig. 2 (a) is the curve in $C = F^{\text{tot}}$. 

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FIG. 1: Dependence of the fluorescence intensity and the ratio $I_{\text{sup}}/I_{\text{sol}}$ on spermidine concentration, in the case of YO-PRO-1 (a) and YOYO-1 (b). ●: Fluorescence intensity of the solution, ○: Fluorescence intensity of the supernatant, and ■: the Ratio of the fluorescence intensity of the supernatant ($I_{\text{sup}}$) and the solution ($I_{\text{sol}}$).
FIG. 2: Dependence of the fluorescence intensity on spermidine concentration with theoretical curves. We obtained $\alpha$ and $n$ by the multiparameter fits using Eq. (7). The values of $x_c$ were given and the fits were performed within each range, $x < x_c$ and $x > x_c$. The optimal fits were obtained by $x_c = 0.14$ mM for YO-PRO-1 and 1.2 mM for YOYO-1. (a) YO-PRO-1: $x_c = 0.14$ mM; $\alpha = 2.2 \times 10^2$ and $n = 1.2$ for $x < x_c$ (solid line); $\alpha = 0.8 \times 10^2$ and $n = 1.8$ for $x > x_c$ (dotted line). (b) YOYO-1: $x_c = 1.2$ mM; $\alpha = 1.7 \times 10^4$ and $n = 1.6$ for $x < x_c$ (solid line); $\alpha = 0.43 \times 10^4$ and $n = 1.4$ for $x > x_c$ (dotted line).
FIG. 3: Schematic diagram of DNA condensation in the presence of dyes (ellipse) and spermidine (circle). The dyes are exchanged for spermidine with the increase of the spermidine concentration, and DNA condensation occurs when \( \Phi \) reaches \( \Phi^* \) at \( x = x_c \).