Higher Order Structure of DNA Controlled by the Redox State of Fe^{2+}/Fe^{3+}

Yuichi Yamasaki and Kenichi Yoshikawa*

Contribution from the Graduate School of Human Informatics, Nagoya University, Nagoya 464-01, Japan

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Abstract: We performed fluorescence microscopic observation of the conformational change of individual T4DNAs (166 kbp) induced by Fe^{2+}/Fe^{3+}. Individual DNAs undergo a marked discrete transition from an elongated coil into a collapsed globule with an increase in the Fe^{3+} concentration at around 1−2 μM. On the other hand, DNAs remained in the elongated coil state with the addition of Fe^{2+} up to a concentration of 30 μM. Using these experimental results we tried to control the transition of DNA by the redox reaction of Fe^{2+}/Fe^{3+}. We found that collapsed globule DNA unfolds with the reduction of Fe^{3+}. The results have been analyzed theoretically in terms of the double minima in the free energy profile of a single DNA molecule, indicating that the change in the translational entropy of the counterions is the main reason why high-valency ions are more effective in inducing the collapse.

Introduction

DNA is a polyelectrolyte with a high density of negative charge. Thus, an aqueous solution is generally a good solvent for DNA. Since the persistence length of duplex DNA[1−4] is on the order of 500 Å and the distance between the base pairs[5] is 3.4 Å, a DNA chain larger than about 1000 base pairs (kbp) behaves like a flexible chain, i.e., long DNA chains usually exhibit an elongated coiled state in an aqueous environment in vitro. More than a decade ago, Yanagida et al. showed that in the aqueous phase, long DNA chains are actually present as thermally fluctuating coiled chains, by the direct observation of conformation in fluorescence-labeled individual DNAs by fluorescence microscopy.[6,7] On the other hand, DNA chains exist as very compact globules in both prokaryotic and eukaryotic living cells.[8] For example, in individual human cells, DNA chains with a total length on the order of a meter are contained in a small space about the size of a micrometer.[5] Currently, the change in the structure of compact folded DNAs has attracted the interest of biologists, since this change in the higher order structure of DNAs is expected to be closely related to the mechanism of the self-regulation of replication and transcription in living cells.[9,10] Thus, physicochemical studies of the large structural changes of long DNAs are expected to shed light on this important problem in biology.

It has been shown[11] that cations with a valence of ≥3 are generally required to induce the condensation of DNAs in aqueous solution at room temperature. Among the multivalent cations, spermine(4+),[12] spermidine(3+),[13] and hexaammineco-balt complex, $Co(NH_3)_6^{3+}$,[14] have often been used to study DNA condensation with the help of methods such as electron microscopy, light-scattering, sedimentation, or other spectroscopic methods.[11,15] In previous reports, however, the term "condensation" did not seem to be defined rigorously, i.e., this term was used to indicate the condensation of DNAs without definitely distinguishing between the collapse of a single molecule and multimolecular aggregation.[11] This may be due to the difficulty of measuring individual isolated DNAs with currently available experiment tools. Under these conditions, the transition of elongated DNAs into a "condensed state" has been reported to be continuous rather than discrete.[16−19] Very recently, contrary to this traditional view of the continuous nature of the "condensation" of DNA, it has become clear that individual DNAs exhibit an all-or-none transition, i.e., a first-order phase transition, whereas the ensemble average appears to be continuous.[20] This discovery regarding the marked discrete transition was performed by observing single DNA molecules through the use of fluorescence microscopy, which makes it possible to clearly distinguish between the collapse of a single molecule and multimolecular aggregation through the direct observation of individual DNAs.[20−25]

* Author to whom all correspondence should be addressed.

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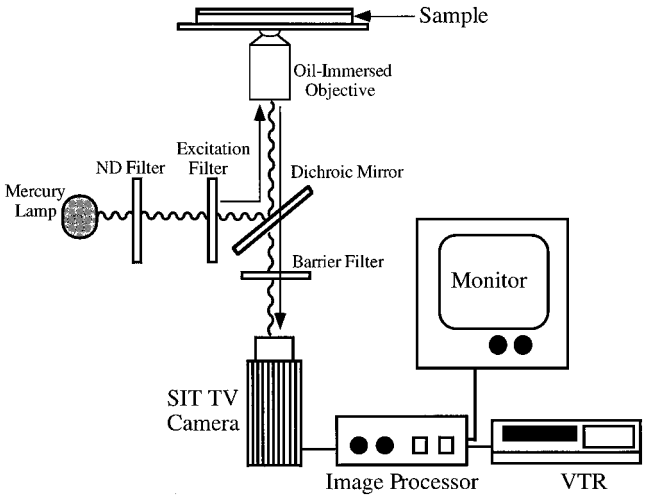

Figure 1. Schematic diagram of the experimental apparatus.

In the present study, we examined the effect of the redox reaction of Fe^{2+}/Fe^{3+} on the higher order structure of DNA by using fluorescence microscopy to investigate the conformation of isolated long duplex DNAs. Fe^{3+} at a concentration on the order of 1 μM induces a large discrete transition of individual long DNAs. In contrast, Fe^{2+} up to a concentration of 30 μM has essentially no effect on the higher order conformation on DNAs. This result prompted us to try to control the higher order structure of DNA by the redox reaction of Fe^{2+}/Fe^{3+}.

Experimental Section

Materials. Bacteriophage T4DNA (166 kilobase pairs with a contour length of 57 μm)[1,2,26] was purchased from Nippon Gene. Iron(II) chloride tetrahydrate, iron(III) chloride hexahydrate, and the fluorescence dye 4′,6-diamidino-2-phenylindole (DAPI)[27] were obtained from Wako Pure Chemical Industries Ltd. and used without further purification.

Sample Preparation. Sample solutions were prepared according to the following procedure. DNA molecules were diluted with Tris−HCl buffer solution (10 mM Tris, 10 mM NaCl, pH 7.2) containing the fluorescent dye. Buffer solutions with a desired amount of metal ion were mixed with the DNA buffer solution. The final concentrations (DNA in nucleotide; 0.06 μM, DAPI; 0.06 μM) were kept constant throughout the fluorescence microscopic experiment. Under these conditions, the number of DAPI molecules per base pair is estimated to be 0.05, and both the contour length and the persistence length remain nearly the same as in the absence of DAPI.[1,2] All solutions were prepared with Millipore water (18.3 GΩ). To avoid the oxidation of Fe^{2+} by atmospheric oxygen, Fe^{2+} was treated in O_2-free solution that had been bubbled with N_2 for 30 min, and preparations were carried out in a glovebox under a N_2 atmosphere. Since Fe^{3+} is known to induce hydrolysis, which leads to oligomerization in neutral solutions,[28] stock solutions of Fe^{3+} were adjusted to pH 1.0 and prepared fresh before each experiment.

Fluorescence Microscopy. Fluorescence microscopic observation[6,7] was performed as follows. DAPI molecules in the sample solutions were excited by 365-nm UV light, and fluorescence images of individual DNA chains were observed with use of a Carl Zeiss Axiovert 135 TV microscope and recorded through a Hamamatsu SIT TV camera and an image processor (Argus 10, Hamamatsu Photonics). A schematic diagram of the direct observation system is shown in Figure 1. The observation was carried out at room temperature, ca. 20 °C. It has been confirmed that the equilibrium size distribution in DNAs is attained at least 3 h after sample preparation. In the present paper, we will show only the data after the equilibriation. The apparent length of the long axis, *L*, which was defined as the longest distance in the outline of a DNA image, was evaluated with an image processor. Due to the blurring effect of fluorescence light, DNA images were slightly thicker than the actual chains by about 0.3 μm.[1,2,29,30] The diffusion coefficient for each DNA molecule was evaluated from the mean square displacement of the fluorescence images.[1,2,29,30] Special care was taken to clean the glass microscope slides and coverslips thoroughly before each observation. They were treated at 500 °C for 1 h, soaked in hydrogen peroxide for more than 1 day, immersed in ethanol for more than 12 h, and rinsed with ethanol. Finally, they were dried at 35 °C for more than 30 min.

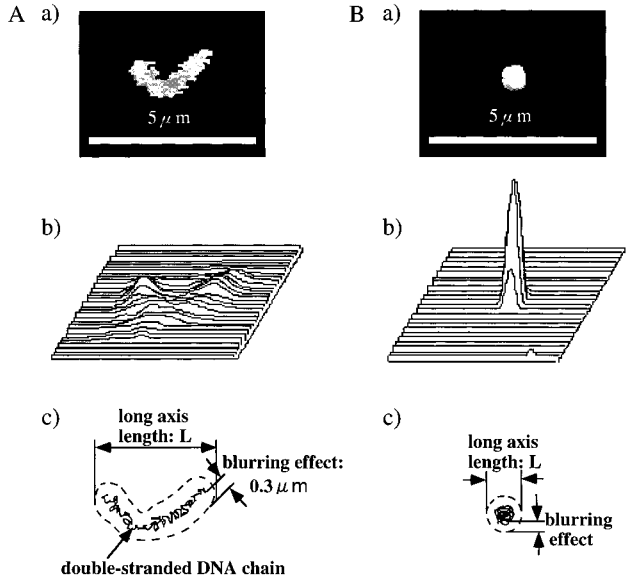

Figure 2. Coiled (A, left) and globular T4DNA (B, right) at the same concentration of Fe^{3+} (1.5 μM) in the coexistence region (see Figures 3 and 4). (a) Fluorescence microscopic images of T4DNA. The full stretched length, or the contour length, of T4DNA is 57 μm.[1,2] (b) Fluorescence light intensity distributions for the images given in part a. The integrated fluorescence intensity was found to be essentially the same for coil and globule DNAs. (c) Schematic representation of the fluorescence images and the corresponding conformations in a single double-stranded DNA molecule.

Electron Microscopy. A JEOL EM1200EX microscope was used at an accelerating voltage of 100 kV. EM grids were rinsed with acetone and then dipped in an isoamylacetate solution of 2% collodion and allowed to dry on blotting paper. A thin layer of carbon was shadowed onto freshly cleaved mica, and then floated off the mica onto a clean surface of water. Collodion-treated grids were placed upside down on the carbon layer and then picked up, yielding very clean carbon-coated grids. Carbon-coated grids were rendered hydrophilic by glow discharge in vacuo. One drop of sample solution was deposited on the grid and after 1 min was stained with an aqueous solution of 2% uranylacetate for 30 s.

Results

The upper panels in Figure 2 show fluorescence images of T4DNA molecules in (A) the elongated coiled state and (B) the collapsed globule state, observed in Tris−HCl buffer solution with 1.5 μM of Fe^{3+}, where coil and globule DNAs coexist in the solution. The spatial distributions of the fluorescence light intensity in these images are given in the middle panels of Figure 2. The schematic representations in the bottom panels of Figure 2 indicate the corresponding conformations in the long duplex DNA molecules together with the effect of blurring. With the

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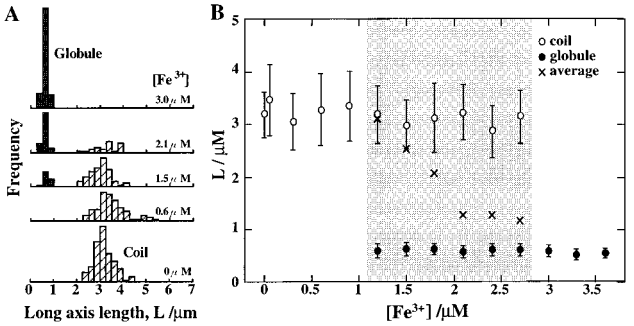

Figure 3. (A) Histogram of the long-axis length, *L*, of T4DNA molecules at various concentrations of Fe^{3+}. Each area of the histogram is normalized to be equal. (B) Long-axis length, *L*, of T4DNA molecules vs the concentration of Fe^{3+}. The open and closed circles indicate the mean value of *L* in coiled and globular DNA molecules, respectively. The vertical bars show the standard deviation. The shaded area shows the region where the coil and globule coexist. The change in *L* for the ensemble average (cross symbols) appears to be continuous, despite the all-or-none nature of the transition in individual DNAs.

addition of Fe^{3+} up to 1 μM, all of the DNAs in the solution remain in the random coil structure and display intramolecular and translational Brownian motion. A further increase in the Fe^{3+} concentration induces the collapse of individual T4DNA molecules into small compact particles, i.e., globules. At intermediate Fe^{3+} concentrations, the coil and globule states coexist in the solution.

To characterize the conformational transition of DNA induced by Fe^{3+} in a quantitative manner, a series of measurements were carried out by changing the Fe^{3+} concentration from 0.06 to 3.5 μM. As a parameter of the effective size, the long-axis length *L* of DNA macromolecules (see Figure 2) was measured directly from the video image. The results are given as a histogram in Figure 3A, which shows the distribution of the long-axis length *L* at different Fe^{3+} concentrations (at least 100 DNA molecules were measured at each concentration). This figure indicates that when the concentration of Fe^{3+} is lower than 0.6 μM, the maximum value of *L* is about 3 μm and the distribution is rather wide, i.e., all of the DNAs are in the random coil state. On the other hand, when the Fe^{3+} concentration is above 3 μM, the maximum value of *L* is less than 1 μm with a narrow distribution width, i.e., all of the DNAs are in the collapsed globule state. Between these limits lies the intermediate case, at which bimodality is observed in the *L* distribution, indicating the coexistence of the coil and globule states. It should be noted that, in the actual measurement, discrimination between coil and globule is easy by using successive video frames based on the observation of intramolecular thermal fluctuation. The coiled DNAs exhibit remarkable time-dependent conformational changes, whereas the globules remain as bright light spots accompanied by translational thermal motion that is much more significant than that in coils.

Figure 3B shows the dependence of the long-axis length *L* on the Fe^{3+} concentration. The open and closed circles indicate the mean values of *L* for the coil and globule states, respectively, and the vertical bar is the width of the distribution given as the standard deviation. The shaded region corresponds to coexistence of the coil and globule states. It is clear that individual DNAs exhibit large discrete conformational changes, i.e., a first-order phase transition. On the other hand, the average value of *L* of the ensemble of DNAs exhibits a continuous transition, as indicated by the cross symbol in Figure 3B. Thus, the discrete nature of the coil−globule transition disappears in the ensemble of DNAs. This feature of the transition explains why most previous experimental studies[16−19] of the collapse transition of

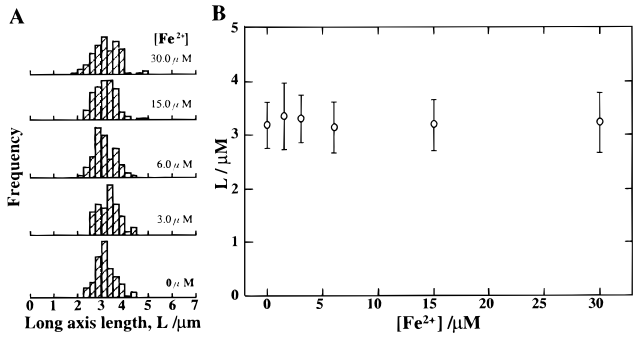

Figure 4. (A) Histogram of the long-axis length of T4DNA molecules at various concentrations of Fe^{2+}. (B) Long-axis length of T4DNA molecules vs the concentration of Fe^{2+}. All DNA chains remained in the coiled state at the concentrations tested.

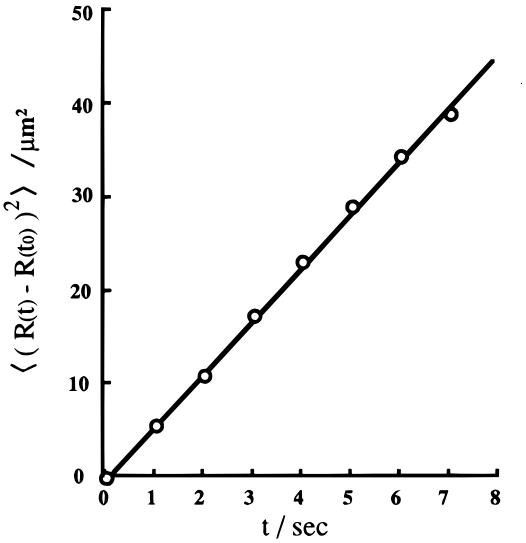

Figure 5. The translational thermal motion of a globular DNA as given by the time dependence of the mean-square displacement at $[Fe^{3+}] = 3$ μM.

polyelectrolytes including DNA have reported that the transition is continuous in nature.

Figure 4 shows a histogram (part A) and the mean value of *L* (part B) as it varies with the concentration of Fe^{2+}. In contrast to the result with Fe^{3+}, all of the DNAs remain in the coil state up to at least 30 μM Fe^{2+}.

Next, we tried to evaluate the actual size of globular DNAs by avoiding the effect of blurring in the fluorescence images. We measured the time-dependent translational displacement of individual globule DNAs complexed with Fe^{3+}. With the sample solution tightly sealed between glass plates, it is possible to minimize convective flow. Figure 5 shows a plot of the mean-square displacement versus time. Using the slope of this relationship, a diffusion constant was obtained for individual globule DNAs based on the following relationship:[1,2,29,30]

$$\langle(\mathbf{R}(t) - \mathbf{R}(0))^2\rangle = 4Dt \qquad (1)$$

where $\mathbf{R} = (R_x, R_y)$ is a two-dimensional vector indicating the spatial position of the globule. The hydrodynamic gyration radius R_H was calculated from *D* based on the Stokes−Einstein relation according to the Zimm model[25] as follows:

$$R_H = k_B T/6\pi\eta_s D \qquad (2)$$

where k_B is the Boltzmann constant and η_s is the viscosity of the solvent. With use of this procedure, the hydrodynamic radius R_H for the globule state was evaluated to be 65 ± 6 nm.

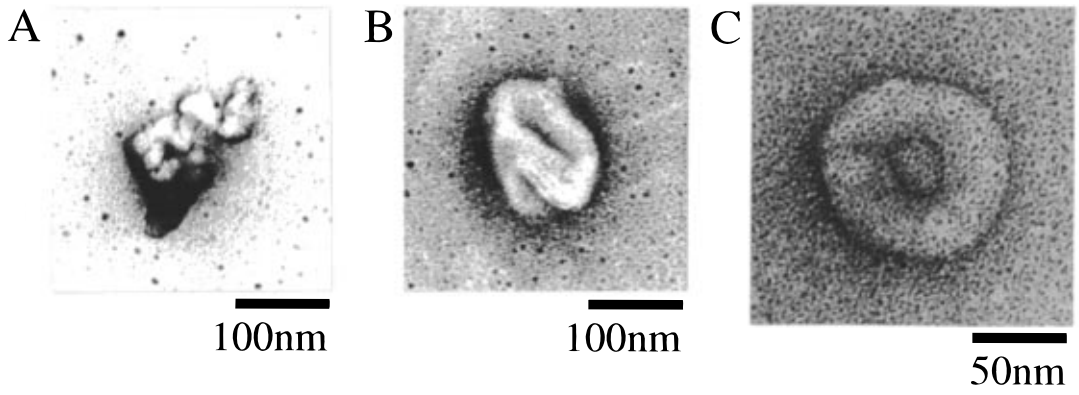

Figure 6. Transmission electron micrograph of globular T4DNA. Parts A and B were formed with Fe^{3+}, and part C was formed with $Co(NH_3)_6^{3+}$.[25]

To obtain the information on the structure of the DNA/Fe^{3+} complex, we observed DNA complexes using transmission electron microscopy. Parts A and B of Figure 6 indicate that DNA/Fe^{3+} complexes form a compact structure with a morphology that is markedly different from the toroidal structure[32−34] of the DNA/$Co(NH_3)_6^{3+}$ complex (Figure 6C). The diameter of DNA/Fe^{3+} complexes by electron microscopy is 100−120 nm, which corresponds well to the hydrodynamic radius of 65 nm estimated from the diffusion constant of individual DNAs determined by fluorescence microscopy.

On the basis of our findings regarding the large difference in the effect of Fe^{2+}/Fe^{3+} on the higher order structure of DNA, we tried to control the structure of DNA by changing the redox state of the ions. To trace the conformational transition of DNA chains upon the reduction of Fe^{3+} to Fe^{2+}, fluorescence microscopic observation was performed under a spatial gradient of ascorbic acid. A DNA sample in 3.5 μM Fe^{3+} solution without ascorbic acid was placed in the middle of a glass slide, and a drop of 18 μM ascorbic acid solution was placed on the border of a coverslip. The unfolding of a globular molecule under diffusional flow of the reductant is shown in the series of photographs in Figure 7. It is clear that a globular DNA complex with Fe^{3+} unfolds under the influence of ascorbic acid. We found that the folding of a coil into a globule is also possible by the oxidative reaction of Fe^{2+}. However, in this oxidative reaction, unfolded DNAs in the coil state were soon cut into small fragments. To induce the folding of DNA chains without any fragmentation by oxidation, optimization of the conditions of oxidation is important.

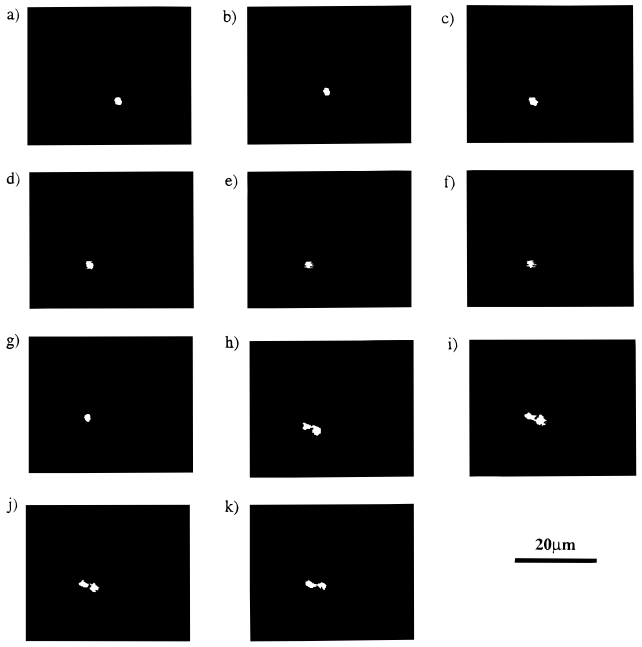

Figure 7. Unfolding of globular DNA into a coiled state by the reduction of Fe^{3+} to Fe^{2+} with the addition of ascorbic acid, as observed by fluorescence microscopy. The interval between the frames is 0.5 s.

Discussion

Double-strand DNA is a stiff polymer with a persistence length l on the order of 500 Å,[1−3] corresponding to ca. 150 base pairs. Thus, an individual synthetic oligomer of DNA, up to 100−200 bps, behaves just like a rigid rod. On the other hand, living cells generally possess giant DNAs. Even in simple bacterial cells, DNA chains are on the order of 10^6 bps. The conformation of such a long coiled polymer with a contour length L can be approximated[35] as a succession of rod-like segments of length 2l that are freely joined to each other, where the number of segments is L/2l. Thus, individual long DNA from living cells behaves as a flexible coiled chain in an aqueous environment in the absence of condensing agents. In the present study, we examined the higher order structure of such "flexible" long DNA chains. Therefore, it should be noted that the discrete transition shown in Figure 3 will never be encountered for oligomeric DNAs with on the order of 10^2 bps. In a comprehensive study of the "condensation" of DNAs, Bloomfield concluded that in aqueous solution a cation with a valence ≥3 is necessary to cause DNA condensation, with the exception that Mn^{2+} produces the condensation of supercoiled plasmid DNA, but not of linearized plasmid.[11] To explain this experimental trend, it has been suggested that DNAs condense in the presence of multivalent cations when 89−90% of the negative charge is neutralized. On the basis of an estimation of the degree of counterion condensation, it has been regarded that divalent cations cannot neutralize DNA up to 89−90%. On the other hand, it has recently been found that divalent cations, such as diaminopropane, can induce the collapse of single DNAs when their concentration is rather high, around 20 mM.[25] On the basis of theoretical considerations, it has been suggested that the free energy of a long DNA chain exhibits double minima in the presence of multivalent cations, including divalent ions: the double minima correspond to the elongated coil and collapsed globule states.[36] Since the degree of counterion

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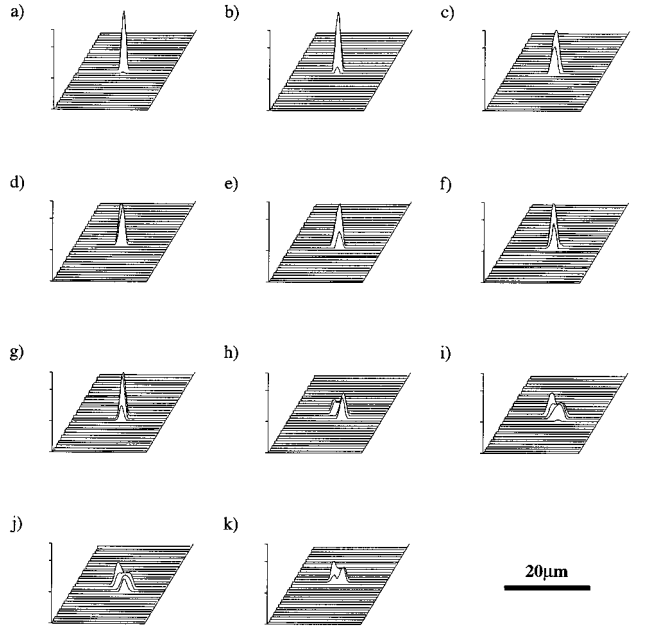

Figure 8. Unfolding process shown by the change in the fluorescence intensity distribution (the same experiment as in Figure 7).

condensation should change between the coil and globule states,[37] it seems to be premature to conclude that the degree of charge neutralization in the coil state controls the transition. Instead, it is important to fully consider the contribution of the free energy, including the conformational free energy of the chain.

Now, let us discuss the conformational change of isolated DNA chains in terms of the free energy. The free energy of a single DNA molecule, F_{total}, can be written as a sum of four components:[36,38]

$$F_{total} = F_{ela} + F_{int} + F_{trans} + F_{elec} \quad (3)$$

where F_{ela} is the free energy of elastic deformation of the DNA chain; F_{int} describes the interaction of DNA segments; F_{trans} is the translational entropy of small ions (i.e., the multivalent ion, its counterion, and the counterion of DNA); and F_{elec} is the electrostatic contribution. As for the free energy of rather long polyelectrolytes, as in T4DNA chains, it is expected that the contribution of F_{elec} is relatively small compared to the other terms.[36,38] In the elongated coil state, the distance between different parts along a single DNA chain is much larger than the Debye length in the usual aqueous environment. As for the collapsed globule state, the negative charge of the phosphate group is almost neutralized with the enhanced counterion binding,[37] indicating that the contribution of F_{trans} is much more significant than that of F_{elec}. Thus, we do not consider F_{elec} in the following discussion for the sake of simplification.

On the basis of a theoretical analysis of the competition between F_{ela} and F_{int} within the framework of mean field approximation, it is evident that there are double-minima in the free-energy profile of a sufficiently stiff polymer. Thus, individual polymer chains undergo a discrete first-order phase transition, between the elongated coil and collapsed globule states. It has been shown that the effective size, or the radius of gyration, S scales as $N^{3/5}$ and $N^{1/3}$ for the coil and globule states, respectively, where N is the number of Kuhn segments.[35]

As an additional characteristic of this transition, there is a region of coexistence between the coil and globule states that depends on an intensive variable, such as the concentration of the multivalent cation, with a relatively large width. Therefore, the ensemble average of the size of the polymer chains appears to be continuous, i.e., there is no discreteness in the ensemble average of the chains including the first- and higher-order derivatives in the transition curve.[20]

Although the coil−globule transition can be discrete as a result of competition between the first and second terms in eq 3, for the coil−globule transition induced by multivalent cations, the third term in eq 3, F_{trans}, should also be important.[36] F_{trans} can be written as a sum of the contributions from inside and outside the effective volume occupied by a DNA chain: F_t^{in} and F_t^{out}, respectively:

$$F_{trans} = F_t^{in} + F_t^{out} \quad (4)$$

$$F_t^{in}/kT = q^{in} \ln(q^{in}/V) + p^{in} \ln(p^{in}/V) + r^{in} \ln(r^{in}/V) \quad (5)$$

$$F_t^{out}/kT = q^{out} \ln(q^{out}/(\Omega - V)) + p^{out} \ln(p^{out}/(\Omega - V)) + r^{out} \ln(r^{out}/(\Omega - V)) \quad (6)$$

where q^{in}, p^{in}, and r^{in} are the numbers of monovalent cations, multivalent ions, and monovalent anions inside the DNA volume, while q^{out}, p^{out}, and r^{out} are the corresponding values for the outside solution. Ω is the space volume per single DNA chain, i.e., the solution volume per single DNA chain. The effective volume of a DNA is given by $V = \alpha^3 V_0$, where V_0 corresponds to the volume of a coiled DNA in the reference state and $\alpha = S/S_0$. S and S_0 are the radius of gyration of a DNA chain and that of a Gaussian chain with the same contour length. The total number of the individual ions (q, p, and r) inside and outside should be kept constant, e.g., $q^{in} + q^{out} =$ constant. On the basis of the condition of total electroneutrality for the region near a "charged DNA chain", the following relationship should hold

$$mp^{in} + q^{in} = Q + r^{in} \quad (7)$$

where Q is the total number of phosphate groups in a DNA chain. The first and second terms in eq 3 are also interpreted as a function of α. The equilibrium-swelling coefficient, α, of a DNA chain is therefore deduced from a minimization of the total free energy F_{total} with respect to q^{in}, p^{in}, and α. As a reasonable assumption, if we neglect the contribution of the counter anions, the free energy can be rewritten as

$$F_{trans}/kT \approx q^{in} \ln(q^{in}/V) + p^{in} \ln(p^{in}/V) + p^{out} \ln(q^{out}/\Omega) + p^{out} \ln(p^{out}/\Omega) \quad (8)$$

We can now evaluate the effect of the translational free energy on the coil−globule transition. As we have mentioned already, a discrete coil−globule transition can be induced as the result of competition between the elastic and interaction terms (see eq 3). Let us denote the effective volumes of coiled and globular DNA as V_c and V_g. If q^{in} and p^{in} do not change so much before and after the transition in comparison with the change in the effective volume, we can roughly estimate the difference in the translational free energy of small ions in globular DNA with reference to that in coiled DNA, $\Delta F_{trans}/kT$. Actually, the change in the number of counterions between

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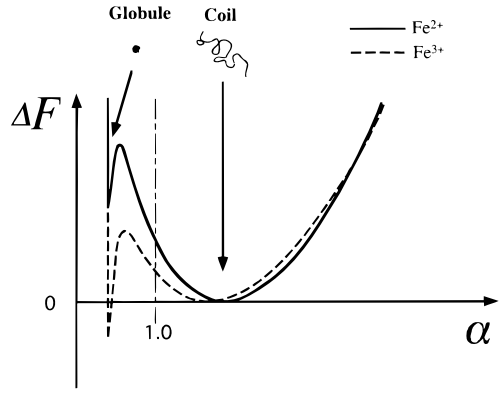

Figure 9. Schematic representation of the change in the bimodal free energy profile in a DNA chain between Fe^{2+} and Fe^{3+}.

the coil and globule states is expected to be at most 20% since more than 80% of the negative charge is already neutralized with counterion condensation in the coil state[11] and the upper limit of counterion binding in the globule is 100%.[39] Although the above assumption will not exactly correspond to the actual coil−globule transition of DNA, a rough estimation of ΔF_{trans} may be useful for understanding the essential aspects of the transition.

$$\Delta F_{trans}/kT \approx (q^{in} + p^{in}) \ln(V_c/V_g) = \{Q - (m-1)p^{in}\} \ln(V_c/V_g) \quad (9)$$

In the above relationship, we have used the condition $\Omega >> V$, in accordance with the experimental conditions in our experiment. The latter relationship in eq 9 can be deduced by using eq 7 where $r^{in} \sim 0$. Since the ratio of the volumes of the coil and globule is 10^3 to 10^4, $\ln(V_c/V_g)$ is +6 to +8. Thus, it is evident that a multivalent cation with a higher valence, or a greater m value, tends to stabilize the globule state. This theoretical expectation corresponds to the experimental trend given in Figures 3 and 4. Equation 9 also predicts that a cation with a higher valency will exhibit a much larger potency for the collapse transition of DNA chains, where free energy difference between globule and coil states is almost linearly dependent on the valency m of the multivalent cation. To demonstrate the effect of Fe^{2+}/Fe^{3+} on the higher order structure of DNA, Figure 9 shows a schematic representation of the change in the free energy profile with different valencies.

In addition to the above effect, the bridging effect of a multivalent cation between DNA segments is also expected to contribute to the collapse of a long DNA chain. It should be mentioned that even with the same valency, the potential to induce collapse will vary depending on the chemical characteristics of the cation, such as the ionic radius and the distance between the cationic centers within a multivalent cation. However, we can conclude here that the most significant effect on the collapse is the change in the translational entropy of the system. A detailed numerical analysis to ascertain this conclusion will be reported in a separate paper.[40]

There are accumulating experimental results on the effect of metal cations on helix−coil (melting) transitions[41] and on supercoiling.[42] These characteristics have been studied mainly for oligonucleotides smaller than several thousand bps. In the present study, we observed the folding and unfolding transitions of 166-kbp T4DNA. It is likely that the folding transition (the higher level in the hierarchy) is closely linked to microscopic structural changes such as supercoiling and the helix−coil transition (the lower level in the hierarchy). Future efforts to clarify the relationship between the high and low hierarchical structures are encouraged. The extension of research along these lines may lead to an understanding of the mechanism of self-regulation in gene expression. In both prokaryote and eukaryote cells, effective transcription of the base-sequence information stored in DNA into RNA should be accompanied by a transition from a helix into a coiled state, and this helix−coil transition cannot proceed unless tightly packed DNAs are, at least partly, unfolded.[43]

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