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Effect of Ethidium on the Formation of Poly(dA)·[poly(dT)]$_2$ Triplex: A Kinetic Study by Optical Spectroscopic Methods

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The kinetics of the formation of triplex poly(dA)·[poly(dT)]$_2$ from poly(dA)·poly(dT) and poly(dT) is examined by various optical spectroscopic methods, including absorption, circular and linear dichroism (LD) spectroscopy. In the pseudo first order condition, where the poly(dT) concentration is kept lower than that of duplex, the association of the poly(dT) is enhanced by the presence of ethidium; the rate constant is proportional to the amount of ethidium in the mixture. When the concentration of the duplex and the single strand is the same, a spectral change is explained by double exponential curves, indicating that at least two steps are involved, the fast association and slow rearrangement steps. In contrast to the pseudo first order kinetics, the association step in an equimolar condition is not affected by the presence of ethidium. In the rearrangement step, the magnitude of LD decreases with an increase in ethidium concentration, suggesting that the bending of polynucleotide around the intercalation site occurs in the rearrangement step.

Introduction

Pyrimidine and purine oligonucleotides can bind to the major groove of duplex DNA at a specific homopurine·homopyrimidine sequence to form triplex DNA. The high selectivity of the third strand makes it an effective method for recognizing a single site in a large duplex DNA. This concept leads to potential biological and therapeutic applications, such as inhibiting gene expression and to designing artificial nucleases.[1] Thus, it is important to investigate the kinetic aspect of the interaction between a homopurine·homopyrimidine duplex DNA and a single stranded DNA which form a triplex. Indeed, there have been several studies, using different techniques, of the kinetics of triplex formation. The techniques included a restriction endonuclease (*AvaI*) protection assay,[2] DNase I footprining,[3] filter binding assay,[3,4] fluorescence resonance energy transfer,[4] UV absorption techniques[5,6] and biomolecular interaction analysis.[7] The association rate constant (k_1) between the oligonucleotides and the target duplex was reported to be 10^3 $M^{-1}s^{-1}$.

We studied kinetics of triplex formation between poly(dT) and poly(dA)·poly(dT) using absorption, fluorescence, and polarized light spectroscopy namely, circular dichroism (CD) and linear dichroism (LD). Although a polynucleotide does not possess any chiral center, it acquires an induced CD spectrum from the excitonic interactions between the electric transition moment of the nucleo-bases.[8] Therefore, CD mainly examines the changes in nucleo-base stacking during triplex formation. The polynucleotide conformation can be examined from the CD of intrinsic polynucleotide absorp-

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tion near 260 nm. The magnitude and sign of the LD signal is affected by orientation and optical factors.[8] The orientation factor reflects the degree of orientation of polynucleotide in the flow; which depends on the contour length and flexibility of the DNA, the flow rate, and the viscosity and temperature of the medium. The optical factor depends on the angle between the transition moment of the nucleobases and the polynucleotide helix axis. Therefore, when conditions are appropriately adjusted, changes in the relative flexibility and contour length of DNA can be studied.

Many intercalators enhance the thermal stability of the Hoogsteen base-paired third strand in the triple helical form of DNA. The intercalator, 9-aminoacridine and its derivatives intercalate to triplex poly(dA)·[poly(dT)]$_2$ and effectively stabilize it.[9] Other intercalators, ethidium and its derivatives[10,11] and benzo[e]pyrido-indole derivatives,[12-14] also enhance the thermal stability of the third strand. However, the effect of intercalators on the kinetics of triplex formation is not known. Therefore, we investigated how the intercalator, ethidium bromide, affects the kinetics of the third strand association. Ethidium bromide was selected as the intercalator in this study because its fluorescence intensity is extremely sensitive to the environment, allowing us to follow the changes in its environment during the triplex formation using fluorescence technique.

Experiments

Materials and Methods. Polynucleotides were purchased from Parmacia and other chemicals from Sigma. A 1 mM sodium phosphate buffer at pH 7.0 containing 1 mM $MgCl_2$ was used throughout this study. The kinetics of the poly(dA)·[poly(dT)]$_2$ triplex formation was detected by spectroscopic signal at 260 nm (for absorption or LD) or at 275 nm (for CD) after an appropriate amount of poly(dT) (M.W.; 7.2×10^4, ε_{264nm}=8520 $M^{-1}cm^{-1}$) was mixed with poly(dA)·poly(dT) (M.W.; 1.6×10^5, ε_{260nm}=6000 $M^{-1}cm^{-1}$). In some cases, ethidium containing poly(dA)·poly(dT) was used as a template duplex in order to observe the effect of the intercalated drug. The fluorescence intensity of a ligand bound to the poly(dA)·[poly(dT)]$_2$ triplex differs from one bound to a poly(dA)·poly(dT) duplex, so we also recorded the change in the fluorescence intensity with respect to time (excitation at 480 nm and emission at 620 nm). A Hewlett Packard 8452A diode array spectrophotometer equipped with a HP80890A peltier temperature controller was used to measure absorption. A Jasco 500C spectropolarimeter was used for CD and a Perkin Elmer LS 50B for the fluorescence measurement.

Linear dichroism (LD) is defined as the differential absorption of light, measured with the polarization vector of the incident light beam oriented parallel and perpendicular to the orientation direction.[8,15] The sign and amplitude of the LD signal depend on both the orientation of the aligned chromophore relative to the reference laboratory axis (optical factor) and an orientation factor reflecting the orientation degree of the sample in the flow. The latter depends on the contour length and flexibility of the DNA, the flow rate, and the viscosity and temperature of the medium. Since the relative angle of the nucleo-bases of the triplex does not change (essentially the nucleo-bases are close to perpendicular to the polynucleotide helix axis), it is reasonable to assume that the change in the LD magnitude upon the triplex formation is primarily due to the orientation factor. When the flow rate is kept constant, the magnitude of the LD signal reflects the contour length and flexibility of the polynucleotide. The LD signal of the oriented polynucleotide was measured on a Jasco 500C spectropolarimeter equipped with an Oxley prism to convert the circularly polarized light into linearly polarized light, as described by Nordén and Seth.[15] The orientation of the polynucleotide was achieved by a flow Couette cell with an inner rotating cylinder. The path length of the light of the Couette cell was 1 mm.

Analysis of the kinetic data. The formation of a triple helical poly(dA)·[poly(dT)]$_2$ from poly(dA)·poly(dT) duplex and single stranded poly(dT) may be described in two steps by:

$$D+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} DS^* \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} T \qquad (1)$$

where D, S, DS* and T represent the poly(dA)·poly(dT) duplex, single stranded poly(dT), intermediate triplex and poly(dA)·[poly(dT)]$_2$ triplex, respectively. The first step is the association of the single strand with the duplex to form the precursor of the triplex. The second step is rearrangement step of nucleo-bases for the precursor to stabilize the triplex.

By adjusting the reaction conditions, the above reaction scheme can be reduced to a pseudo first order reaction. When the reaction is carried out so that (1) the rearrangement of the nucleo-bases is slow and (2) the concentration of the duplex is excess over that of a single strand (10-500 times greater in our experiment), then the concentration of the duplex remains close to its initial concentration as the reaction proceeds, and moreover, (3) if the kinetics are carried out in a temperature where the first equilibrium of the above reaction shiftes to the right ($k_1 \gg k_{-1}$), the rate of the reaction may be described by a simple pseudo first order reaction as[6]

$$[C]=[C]_0 \exp(-k_{obs}t) \qquad (2)$$

where $[C]$ is the concentration and k_{obs} is the pseudo first order reaction constant. Applying the above equation, one can write

$$\ln\left(\frac{P_1-P_\infty}{P_0-P_\infty}\right)=k_{obs}t \qquad (3)$$

where P_0, P_1 and P_∞ are the physical properties of the initial state, at a given time t after mixing the duplex with a single strand, and of all the single strands binding to the duplex completely.

When the same concentrations and volume of poly(dA)·poly(dT) and poly(dT) were mixed, the resulting changes in the spectral properties (P) did not fit into the single exponential curve but could be explained by a sum of two exponentials. A double exponential suggests that the reaction is proceeding through at least two different kinetic steps. When two components were observed, they were referred to as the fast and slow components. We assumed that in the reaction scheme (eq. 1), at the temperature below the triplex melting point, the destabilization step may be negligible (k_1

$\gg k_{-1}$ and $k_2 \gg k_{-2}$).

Results and Discussion

Effect of intercalators in the association step of poly(dT). As the triplex is formed, the absorption spectrum of the poly(dA)·poly(dT) and poly(dT) mixture is changed with time. The mixing was performed at room temperature, which is well below the triplex melting temperature, and the concentration of poly(dA)·poly(dT) excesses over that of poly(dT); therefore, we assumed the condition represented a pseudo first order reaction. The absorbance in the nucleo-base absorption region of the mixture decreases upon triplex formation (Figure 1a, insertion). Decreases in the absorbance at 260 nm, where hypochromicity was the largest, were recorded over time (Figure 1a). The values on the left side of Eq. (3) were plotted with respect to time in order to obtain k_1 (Figure 1a). The resulting plot was a straight line, suggesting that under our conditions the reaction was the first order. From the slope, the pseudo first order rate constant was calculated to be about 3.8×10^{-4} s^{-1}, which is 10 times slower than that reported by Xodo[6] for oligonucleotide. This difference may be attributed to the length of the nucleotide and the stabilization conditions of the triplex. The third strand association was faster in the presence of the intercalator ethidium. The rate constant was roughly proportional to the amount of intercalator in the system (Figure 1b). When the mixing ratio reached 0.1, which is equivalent to 1 intercalator per 10 base pairs, the association of the poly(dT) was faster than that in the absence of ethidium by a factor of 10.

The intercalator ethidium undoubtedly enhances the association of poly(dT). It is therefore conceivable that poly (dT) binds to the region of the duplex where ethidium is intercalated prior to binding to any other regions. Upon ethidium intercalation, the duplex poly(dA)·poly(dT) is unwound and elongated, and consequently the duplex is stiffened. These conformational changes in the duplex may enhance the binding rate. The detailed mechanism behind this conformational change causing enhancement of the third strand may be a subject for further investigation. It is also possible that the poly(dT) strand interacts with the intercalated ethidium first (not directly with the duplex at the first step), and then forms the triplex. In this case, the property of the intercalated drug would be an important factor. We recently found that the thermal stability of the third strand depends on the property of the intercalators. For ex-

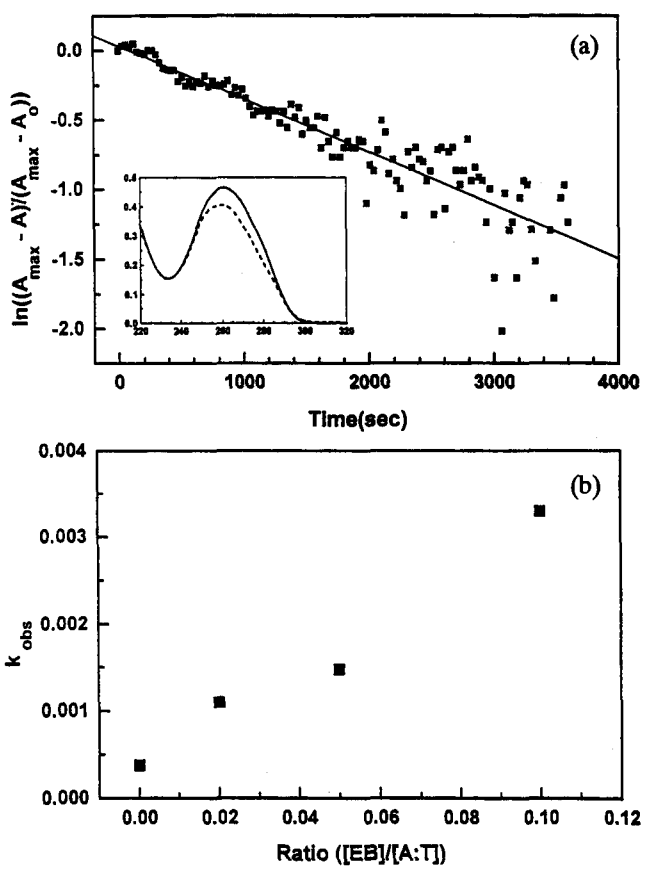

Figure 1. (a) Changes in the absorbance at 260 nm for the poly (dA)·poly(dT) and poly(dT) mixture with respect to time according to the Eq. (3). The rate constant is calculated from the slope to be 3.8×10^{-4} sec^{-1}. The absorption spectrum at the moment of mixing (solid curve) and after triplex formation is shown in the insert. The concentration of poly(dA)·poly(dT) and poly (dT) are 400 μM and 20 μM, respectively. (b) The rate constants in the presence of various concentrations of ethidium. The concentration of the polynucleotide is the same as in (a).

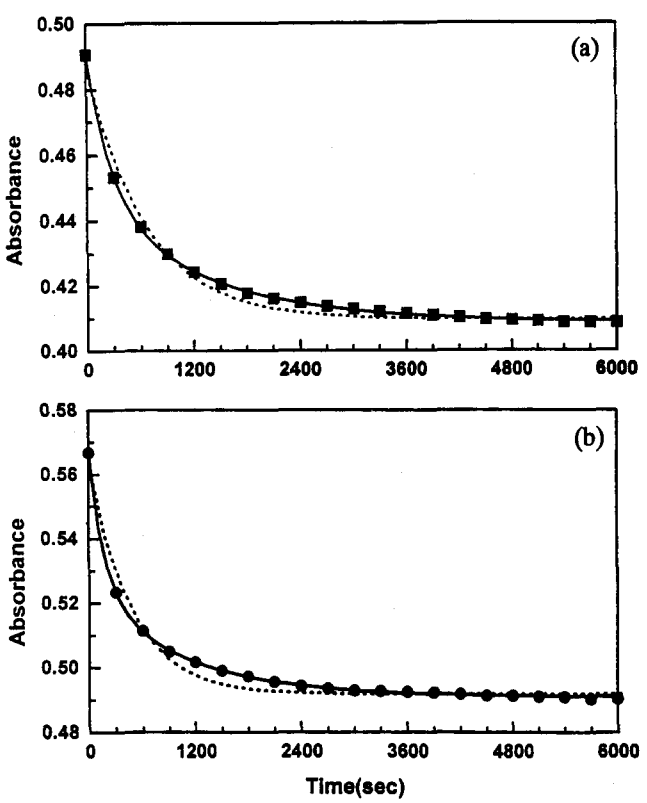

Figure 2. Decreases in the absorbance of the equimolar mixture of poly(dT) and poly(dA)·poly(dT) at 260 nm in the (a) absence and (b) presence of ethidium, with its single exponential (dotted curve) and double exponential (solid curve) fitting curve. The concentration of polynucleotide is 25 μM in base pair for the duplex and in base for the single strand. The mixing ratio of ethidium, relative to the base triplet, is 0.25.

ample, 9-aminoacridine with an aminoalkyl side chain at the 9th position stabilized a poly(dA)·[poly(dT)]$_2$ triplex more efficiently than 9-aminoacridine without a side chain.[16] The kinetic aspects of these drugs are currently under investigation.

Equimolar kinetics detected by absorption, CD and fluorescence. Changes in absorption and CD "see" the stacking and chiral arrangement of the transition moments of the achiral nucleo-bases. The decrease in the absorbance at 260 nm after the mixing of equimolar poly(dT) and poly(dA)·poly(dT) in the absence of ethidium is depicted in Figure 2a and that in the presence in Figure 2b. The mixing ratio was 0.25, at which all possible intercalation pockets of the duplex were saturated by ethidium. The decrease of absorbance was fitted by a single exponential and a double exponential decay. The single exponential curve did not fit satisfactorily to the change in the absorption, but the data fit well to the double exponential decay, suggesting that the formation of triplex proceeds in two different kinetic pathways with different apparent rates. As the concentration of the poly(dT) in the mixture was increased to that of the template duplex, the association rate increased from 3.8×10^{-4} $\mu M^{-1}s^{-1}$ to 3.4×10^{-3} $\mu M^{-1}s^{-1}$, which coincides with the increase by ethidium at a mixing ratio of 0.1 in the pseudo first order condition. In contrast to the absorption change for the pseudo first order reaction, a long-lived decrease in absorbance was observed, which indicated that a simple association and dissociation scheme can not be applied to our conditions. Based on the linear dichroism results (see below), this slow step may be explained as a rearrangement of the triplex. This slow step was not visible in the pseudo first order condition, probably because the change is too small.

The CD of the mixture of duplex poly(dA)·poly(dT) and poly(dT) and that of the triplex poly(dA)·[poly(dT)]$_2$ are appeared to be different due to the regular stacking of the third strand. The CD spectra of the equimolar mixture of poly(dA)·poly(dT) and poly(dT) at the time of mixing and after the triplex was formed are depicted in the Figure 3a (insertion). The formation of the triplex was detected by a CD change at 255 nm and is shown in Figure 3a in the absence of ethidium and that in the presence in Figure 3b, with the single and double exponential fitting. The curve is satisfactorily fitted into a double exponential decay with k_1 and k_2 values of 2.4×10^{-3} $\mu M^{-1}s^{-1}$ and 2.6×10^{-4} $\mu M^{-1}s^{-1}$ in the absence of ethidium and 2.2×10^{-3} $\mu M^{-1}s^{-1}$ and 2.7×10^{-4} $\mu M^{-1}s^{-1}$ in its presence. These values are in the same range as those observed in the absorption measurement. The k_2 value for the equimolar mixture was about ten times slower than that for k_1, which is also in agreement with the absorption measurement.

The significant increase in the fluorescence intensity of ethidium after binding to duplex DNAs is well documented. Dehydration of the water shell around the ethidium molecule is generally believed to be the reason for the increase in the fluorescence intensity; upon intercalation, the water shell, which causes the quenching of the ethidium fluorescence, is stripped off. It has been reported that fluorescence of the poly(dA)·[poly(dT)]$_2$ triplex increases more than that of the duplex.[11] Further increases in the fluorescence intensity can be attributed to additional blocking of

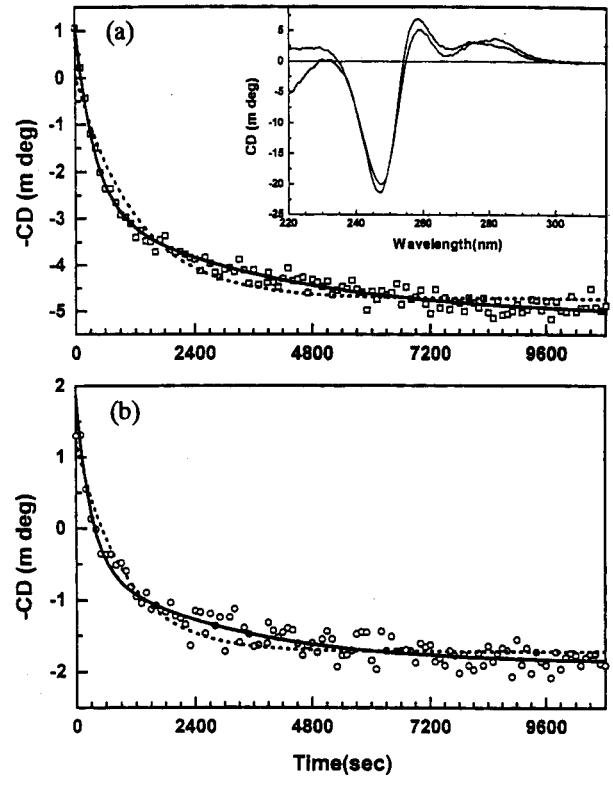

Figure 3. Changes in the CD at 275 nm with time. The curve assignment and conditions are the same as in Fig. 2.

the incoming water molecule by the third strand, which is located in the major groove of the duplex. This observation was confirmed in our experiment (insertion in Figure 4) and used to examine the change in the environment of the intercalator upon triplex formation. Once again, the increase in the fluorescence, with respect to time, was satisfactorily fitted by only two exponential curves, with k_1 and k_2 values of 6.3×10^{-3} $\mu M^{-1}s^{-1}$ and 1.3×10^{-3} $\mu M^{-1}s^{-1}$ (Figure 4).

Equimolar kinetics detected by linear dichroism (LD). After adjusting the experimental conditions and assuming that the nucleo-bases were mostly perpendicular to DNA helix axis, we could infer that the change in the LD

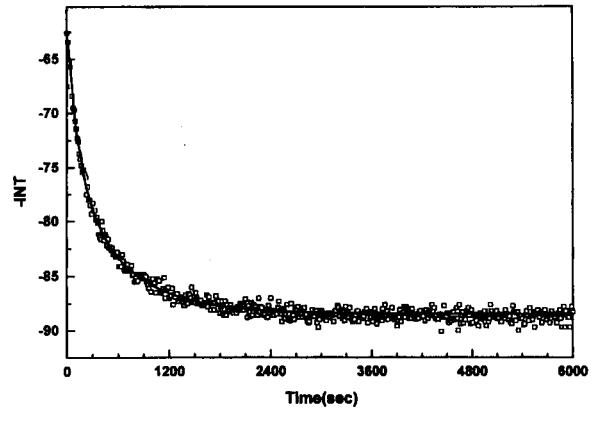

Figure 4. Increases in the fluorescence intensity of intercalated ethidium upon triplex formation. Emission at 620 nm and excitation at 480 nm. See the legend for Fig. 2 for the other conditions.

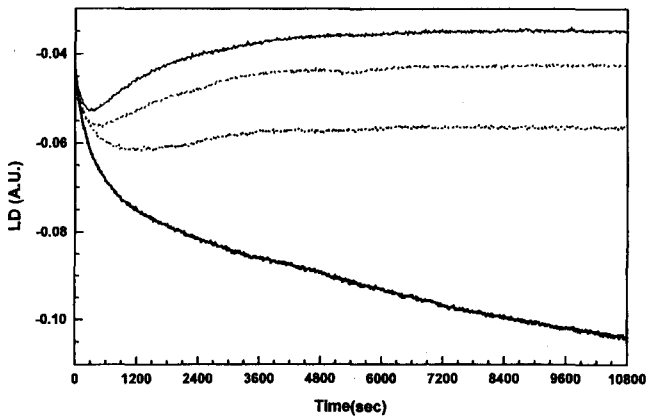

Figure 5. Change in the magnitude of LD at 260 nm as a function of time in the absence and presence of ethidium. The concentration of polynucleotide is the same as in Fig. 2. From bottom, the mixing ratio of ethidium relative to the base pairs of the duplex is 0.00, 0.10, 0.25 and 0.30.

magnitude represented a change in the contour length and/or the flexibility of the polynucleotide. The changes in the LD magnitude at 260 nm, as a function of time in the absence and presence of various amounts of ethidium, are depicted in Figure 5. The LD signal of the single stranded poly (dT) was negligible under the same conditions, indicating that the changes in the LD magnitude were due purely to the triplex formation. When ethidium was absent, the magnitude of the LD continuously increased with k_1 and k_2 values of 3.3×10^{-3} $\mu M^{-1}s^{-1}$ and 1.0×10^{-4} $\mu M^{-1}s^{-1}$, indicating that the polynucleotide was elongated and stiffened upon triplex formation. The k_1 was in the same range as those observed from the absorption and CD and the k_2 was somewhat slower. The first step, which was accompanied by hypochromism in absorption, a change in CD, and increases in the LD magnitude, may be attributed to the association of the poly(dT) to the template poly(dA)·(dT) duplex. Therefore, in the association step, the enhancement in the stacking interaction between the nucleo-bases of the third strand resulted in stiffening and/or elongation of the polynucleotide. The rearrangement step, with small additional decreases in the absorbance and increases in LD magnitude, followed the association.

When the duplex contained ethidium, the decrease in the LD magnitude in the slow step was apparent. As the ethidium concentration increased, this effect was more pronounced, indicating that the decrease in the LD magnitude in the slow step was due to the intercalated ethidium. In the rearrangement step the stacking interaction between the nucleo-bases was enhanced in the presence as well as absence of ethidium (as evidenced by the absorption and CD changes), which was expected to contribute to the increases in the LD magnitude. However, despite the increase in the stacking interaction, we observed surprising decreases in the LD magnitude. Therefore, the triplex may be bent in the rearrangement step near the ethidium intercalation region.

Mechanism of triplex formation. In the pseudo first order condition, ethidium clearly enhanced the association of poly(dT) to poly(dA)·(dT) to form the triplex. The rearrangement step was not observed in this condition. However, in the equimolar mixture of the single stranded poly(dT) and duplex poly(dA)·poly(dT), the kinetics of the triplex formation can be explained by two exponential components. The fast component is assigned to the association step and the slow step to rearrangement step. In the equimolar mixture, the association step was not affected by the presence of ethidium, which is in contrast to the pseudo first order condition. The enhancement effect of the ethidium may be overwhelmed by the fast association induced by the highly concentrated poly(dT). The slow step was characterized by an additional hypochromism in the absorbance and an increase in the LD magnitude. In the slow step, therefore, nucleo-bases probably rearrange themselves, resulting in more stacking between them. However, the presence of ethidium induced a decrease in the LD magnitude, indicating that the polynucleotide becomes flexible or bent, while the enhancement of the stacking interaction was evidenced by the increased absorbance and CD. Therefore, in the ethidium intercalated region, the bending of the triplex in the rearrangement step may be induced without disturbing the stacking of the third strand, resulting in additional hypochromism and a reduced LD magnitude at the same time.

Conclusion

The formation of the triplex poly(dA)·[poly(dT)]$_2$ by mixing poly(dA)·poly(dT) duplex and single stranded poly(dT) essentially consists of relatively fast association and slow rearrangement steps, and an intercalator, ethidium, significantly enhanced the rate of the association of poly(dT). The presence of ethidium contributes to decreases in the LD magnitude in the second step conceivably by polynucleotide bending around the intercalation site.

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Interaction between Norfloxacin and Single Stranded DNA

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We compared various spectroscopic properties of a norfloxacin-single stranded DNA complex with those of norfloxacin-double stranded DNA complex. Norfloxacin binds to both double- and single stranded DNA, and we observed the following spectroscopic changes for both complexes: hypochromism in the norfloxacin absorption region in the absorption spectrum, the characteristic induced CD spectrum consisting of a weak positive band at 323 nm and a strong positive band at 280-300 nm followed by a negative band in the 260 nm region, a strong decrease in the fluorescence intensity and a red-shift in the fluorescence emission spectrum, and shorter fluorescence decay times. These results indicate that the environments of the bound norfloxacin in both DNAs are similar, although the equilibrium constant of the norfloxacin-single stranded DNA was twice as high as the norfloxacin-double stranded DNA complex. Both complexes were thermodynamically favored with similar negative ΔG^{o}. Negative ΔH^{o} terms contribute to these spontaneous reactions; ΔS^{o} term was unfavorable.

Introduction

Quinolones are synthetic antibacterial agents that have been widely studied in academic and industrial laboratories.[1-5] Considerable biological data indicates that type II DNA topoisomerase, a DNA gyrase,[6-17] is the functional target of the inhibital action of these drugs. Type II topoisomerase catalyzes the conversion of relaxed supercoiled DNA into a negatively supercoiled form and is essential for cellular life. Shen *et al.* reported that norfloxacin, one of the most potent DNA gyrase inhibitors of the quinolone family, does not directly bind to DNA gyrase but to DNA.[18] Following this discovery, norfloxacin was observed binding to supercoiled, relaxed, and double- and single stranded-DNAs by equilibrium dialysis and a membrane ultrafiltration method.[18-23]

Two binding modes of quinolone to DNA have been proposed.[21,24] In the presence of gyrase and non-hydrolyzable ATP analogue, gyrase induces a specific single stranded DNA pocket in which norfloxacins are bound and stabilized by hydrogen bonding, π-π stackings of the norfloxacin rings, and tail-to-tail hydrophobic interactions.[21] In contrast, norfloxacin reportedly bound to plasmid DNA in the presence of an appropriate amount of Mg^{2+} but exhibited no interaction in either the absence or excessive amount of Mg^{2+} ions when observed by fluorescence technique, electrophoretic DNA unwinding, and affinity chromatography techniques.[24] Based on these observations, a model for the ternary complex, in which the Mg^{2+} ion acts as a bridge between the phosphate groups of nucleic acids and the carbonyl and carboxyl moieties of norfloxacin, has been proposed.

In spite of the above reported binding modes, the interaction of quinolone antibiotics and DNA is not yet clearly understood. For example, we recently found that one of the fluorescent quinolones, norfloxacin, can form a complex with double stranded *calf thymus* DNA without ATP or Mg^{2+} mediation; this was determined by a red-shift and hypochromism in the normal absorption, a strong induced circular dichroism, and strong fluorescence quenching in the presence of DNA.[25] Furthermore, based on the linear dichroism measurement, the aromatic molecular plane of DNA-bound norfloxacin was concluded to be close to parallel relative to the DNA bases, which excluded the possibility of a groove binding mode.[25] In this work, we investigated the binding properties of norfloxacin to mixed sequence single stranded DNA using various spectroscopic techniques in the absence of ATP and Mg^{2+}. In particular, we compared the spectroscopic properties, including the absorption, fluorescence emission, induced CD, and thermodynamic parameters, of a norfloxacin-single stranded DNA complex with those of a norfloxacin-native double stranded DNA complex. This study may be an important key to und-

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