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Use of Fluorescein-Labeled Oligonucleotide for Analysis of Formation and Dissociation Kinetics of T:A:T Triple-Stranded DNA: Effect of Divalent Cations[1]

Christine Ellouze, François Piot, and Masayuki Takahashi[2]

Groupe d'Etude de Mutagénèse et Cancérogénèse, UMR 216, Institut Curie & CNRS, F-91405 Orsay, France

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The formation and dissociation kinetics of homonucleotide oligo(dT):oligo(dA):oligo(dT) triplex have been analyzed by fluorescence measurements of fluorescein-labeled oligo(dT) providing considerably higher sensitivity to monitoring reaction kinetics than traditional hypochromicity and circular dichroism. The triplex is concluded to be formed by a bimolecular process corresponding to the addition of the oligo(dT) strand to a preserved oligo(dT):oligo(dA) duplex. The association rate was found to be faster the higher the divalent cation concentration and depends upon the nature of divalent ions in the following order of efficiency: $Mn^{2+} > Mg^{2+} > Ni^{2+}$, $Ca^{2+} > Ba^{2+}$. The more efficient metal ions for the triplex formation were found also more efficient for the stabilization. The dissociation kinetics of the third Hoogsteen-bound strand was monitored at below melting temperature by chasing the labeled dT strand from the triplex by excess of non-labeled oligonucleotide. The dissociation rate was found to be almost independent of concentration and nature of cation. The thermodynamic stabilization of triplex by cations is thus a consequence of the increased formation rate.

Key words: divalent cations, fluorescence, fluorescence anisotropy, kinetics, triplex DNA.

Triple-stranded nucleic acids have regained particular interest in biology. Triplex DNA has been found to be formed as an intermediate in the homologous recombination reaction (*1-4*). In addition, formation of triplex DNA at promoter region could be exploited for artificial regulation of gene expression for biomedical purposes (*5-8*). Analyses and understanding of mechanism of formation and stability of triplex are entertained (for a review see Ref. *9*). Structural and thermodynamic determinations of triplex have been thus advanced.

Various methods for the analysis of kinetics are also developed. However, the methods, especially those for the analysis of dissociation kinetics, are restricted. CD and absorption signals, usually used for the detection of formation and analysis of stability of triplex, are rather weak. Some other techniques such as gel electrophoresis (*10*), affinity cleavage (*11*), and foot printing (*12*) are more sensitive but restricted in the kinetic analysis because of their lower time resolution. Recently, fluorescence energy transfer measurement and filter binding assay were used (*13, 14*). Rougée and colleagues have developed an ingenious approach to determine the dissociation rate from the denaturation and renaturation patterns of triplex upon heating and cooling (*15*). We have examined here the possibility to follow the formation and dissociation reactions by simple fluorescence measurements of a fluorescein probe attached at the 5′ end of DNA.

Our results show that fluorescein-labeled oligonucleotide is indeed a very sensitive tool for studying triplex formation and dissociation in solution and real time. The fluorescence intensity is considerably modified, as well as its anisotropy, upon the formation of triplex. Using this type of fluorescence measurements, we have performed quantitative analysis and made the following observations: (1) formation of conventional dT:dA:dT triplex is faster the higher the divalent cation concentration. (2) By contrast, the dissociation rate is not significantly affected by cation concentration.

MATERIALS AND METHODS

Materials—Oligo(dA), oligo(dT), and fluorescein-labeled oligo(dT) (fluorescein is attached at 5′ end) were purchased from Genset. Their size was 36 bases. The concentration was estimated spectrophotometrically using $\varepsilon_{257} = 8{,}600$ $M^{-1} \cdot cm^{-1}$ (in bases) for oligo(dA) and $\varepsilon_{263} = 8{,}520\ M^{-1} \cdot cm^{-1}$ (in bases) for both labeled and non-labeled oligo(dT). Oligo(dT):oligo(dA) duplex was prepared by cooling slowly equimolar mixture of oligonucleotides (1 mM in bases in a buffer containing 10 mM potassium phosphate and 50 mM NaCl) after heating at 90°C for 5 min.

Experimental Conditions—Experiments with Mg^{2+} ion were mainly performed in a buffer containing 15 mM KH_2PO_4 and 5 mM K_2HPO_4 and indicated concentration of divalent ion. Comparison of effect of various divalent ions was carried out in a buffer containing 20 mM Tris-HCl pH 7.4 and 30 mM NaCl.

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[2] To whom correspondence should be addressed at: Institut Curie, Bat. 110, Centre Université Paris-Sud, F-91405 Orsay, France. Fax and Tel: +33-1-69863011

Kinetic Measurements—The formation of triplex was measured at 20°C upon the addition of non-labeled (dA):(dT) duplex to the labeled oligo(dT) solution. The dissociation was followed by chase of the labeled Hoogsteen-bound dT strand from the triplex by addition of 50-fold excess of non-labeled oligo(dT). The dissociation was measured at 30°C because the reaction was very slow at 20°C. The analysis of kinetics and the determination of kinetic parameters were made according to the simple association-dissociation mechanism:

$$(dT)+(dA):(dT)\ \text{duplex} \underset{k_d}{\overset{k_a}{\rightleftharpoons}} (dT):(dA):(dT)\ \text{triplex} \quad (1)$$

Spectroscopic Measurements—Fluorescence and fluorescence depolarization anisotropy were measured in a FP-777 spectrofluorometer (Jasco) equipped with an automatic anisotropy measurement apparatus (ADP301, Jasco). The cell holder was thermostated. The fluorescence and fluorescence anisotropy were observed at 521 nm (bandwidth of 10 nm) upon selective excitation of fluorescein at 496 nm (bandwidth of 5 nm). Anisotropy was averaged over 25 measurements of 0.5 s each. Circular dichroism was measured in a J-710 spectropolarimeter (Jasco). The dissociation of triplex was followed by CD change at 260 nm upon temperature rise of 0.2°C/min from 15°C.

RESULTS

Detection of Triplex Formation by Fluorescence Measurements of Fluorescein-Labeled Oligonucleotide—We first examined the possibility to detect the formation of triplex DNA by fluorescence change of fluorescein probe attached at the 5′ end of the oligonucleotide. Figure 1 shows the fluorescence and anisotropy change of fluorescein-labeled oligo(dT) [denoted as oligo(dT*)] upon step wise addition of oligo(dA). Since magnesium ions are required for the formation of triplex DNA (*9, 17*), the results with and without magnesium ions were compared. In all cases, the addition of a complementary strand, oligo(dA), decreased the fluorescence intensity about 40% and increased the anisotropy value about three times showing that the binding of oligo(dA) to oligo(dT*) strongly affects the fluorescence character (Fig. 1).

In absence of Mg^{2+}, the anisotropy value reached a plateau at a stoichiometry of 1 oligo(dA) per 1 oligo(dT*) (Fig. 1). Addition of excess of oligo(dA) did not modify the fluorescence. This one-to-one stoichiometry corresponds to a formation of oligo(dA):oligo(dT*) duplex. In the presence of Mg^{2+} (4 or 10 mM), the anisotropy value of free oligo(dT*) was slightly larger than in its absence. Upon the addition of oligo(dA) the anisotropy value increased up to an oligo(dA)/oligo(dT*) ratio of 1:2, and decreased upon

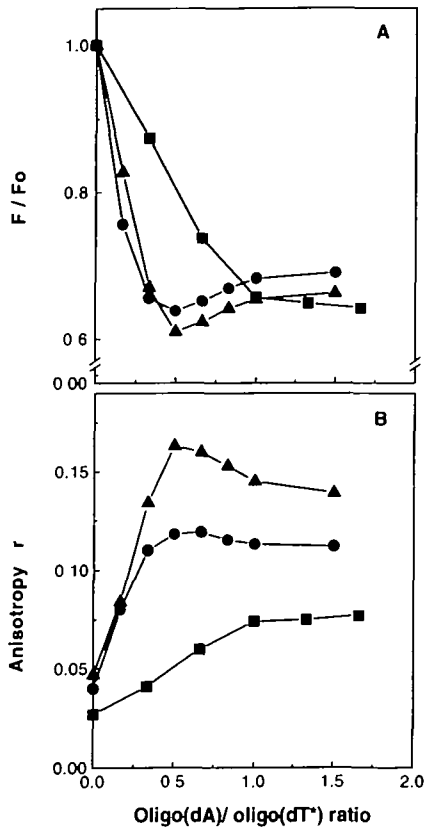

Fig. 1. **Detection of triplex formation by titration of fluorescein-labeled oligo(dT) by oligo(dA).** Oligo(dA) was added in a step-wise manner to 3 μM (in bases) fluorescein-labeled oligo(dT) in absence (■) and presence of 4 mM (●) or 10 mM (▲) $MgCl_2$. After each addition, the fluorescence intensity (panel A) and anisotropy (panel B) of fluorescein ($\lambda_{ex}=496$ nm and $\lambda_{em}=521$ nm) were measured as described in text. The fluorescence intensity was normalized to that of free oligo(dT*).

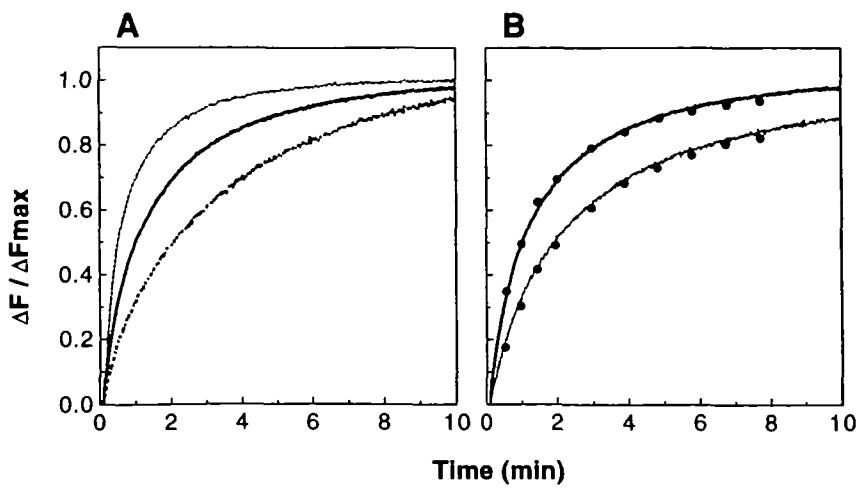

Fig. 2. **Kinetic analysis of dT:dA:dT triplex formation.** Fluorescein-labeled oligo(dT) (3 μM in bases) was added to equimolar oligo(dT):oligo(dA) duplex (3 μM in base pairs) solution and formation of dT:dA:dT triplex was followed by fluorescence change of fluorescein as in Fig. 1. In panel A, the effect of $MgCl_2$ concentration was examined. The experiments were performed at 2 mM (dotted line), 5 mM (solid line), and 10 mM (small dots) $MgCl_2$. In panel B, the effect of oligonucleotide concentration was examined. The experiments were carried out with 1.5 (thin line) and 3 μM (thick line) each of oligo(dT) and dT:dA duplex. The best fit curves based on a bimolecular mechanism are also shown (•••).

addition of further oligo(dA) (Fig. 1). The one-to-two stoichiometry indicates the formation of oligo(dT*):oligo(dA):oligo(dT*) triplex. The decrease of anisotropy upon addition of oligo(dA) when exceeding one-to-two ratio probably corresponds to a disproportion of triplex to oligo(dA):oligo(dT*) duplex.

It is conceivable that the anisotropy value, which reflects the mobility of chromophore (*18*), is larger for triplex than duplex DNA because the triplex has a larger mass and is stiffer (*19*). We note that at 10 mM Mg^{2+} condition, the anisotropy increased linearly up to 2:1 ratio while at 5 mM Mg^{2+} condition the increase was not linear and the anisotropy value at 2:1 ratio is significantly smaller than that expected from the original slope (Fig. 1B). At lower Mg^{2+} concentration a part of the molecules is duplex even at 2:1 mixture in contrast to higher Mg^{2+} concentration conditions where almost all molecules are triplex (Fig. 1). This supports higher stability of the triplex at higher Mg^{2+} concentration. The conclusion was also supported by CD measurements of 2:1 oligo(dT)/oligo(dA) mixture at 2 mM and 6 mM Mg^{2+} conditions. The positive CD band, typical of triplex, at about 260 nm was significantly weaker in 2 mM than in 6 mM Mg^{2+} (not shown).

Kinetic Analysis of Triplex Formation—Taking advantage of fluorescence change of fluorescein-labeled oligo(dT) upon triplex formation, we have analyzed the kinetics. Fluorescein-labeled oligo(dT) was added to preformed oligo(dA):oligo(dT) duplex, and the association was followed by the fluorescence change (Fig. 2). The measurements showed that the triplex formation is faster at higher Mg^{2+} concentration (Fig. 2A). Mg^{2+} ion thus affects the formation step of triplex. Variation of the association rate with Mg^{2+} concentration is shown in Fig. 3.

The association kinetics could not be fitted by monoexponential curve and exhibited bimolecular aspect. The data were analyzed according to this mechanism by the analytical method previously described (*20*). A good fit was obtained in all cases (Fig. 2). In order to further confirm this model, we have examined the effect of concentration of oligonucleotides on the formation kinetics (Fig. 2B). The association reaction was slower at lower oligonucleotide concentration as expected. The kinetics with 1.5 μM oligonucleotides was also well fitted with bimolecular mechanism and with a similar rate constant ($3.0\times10^5\ M^{-1}\cdot s^{-1}$ in concentration in oligonucleotide) as the case with 3 μM oligonucleotides ($3.1\times10^5\ M^{-1}\cdot s^{-1}$) (Fig. 2B). Similar result was obtained using a further lower concentration (0.75 μM) of oligonucleotides (data not shown).

Thermally Induced Dissociation of Triplex—The dissociation step was examined by following the fluorescence change of triplex upon raised temperature. Thermally-induced denaturation of dT:dA:dT triplex occurs in two steps (*21, 22*). In fact, the CD measurements on the triplex formed with dT and dA oligonucleotides showed a two-step denaturation (see Fig. 4B). This could correspond to a triplex-to-duplex transition by dissociation of the third strand and, finally, the transition of duplex into single-stranded DNAs. In order to confirm this mechanism, we have selectively monitored the dissociation of the first and the third strand. The first strand should be involved in Watson-Crick duplex formation while the third strand be Hoogsteen bound to the major groove of duplex. We selectively labeled either the first or the third DNA by forming the triplex DNA, respectively, by adding non-

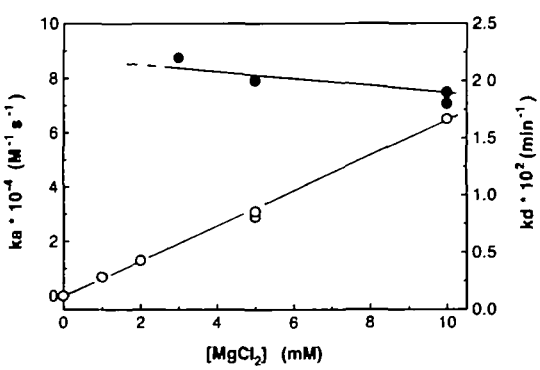

Fig. 3. **Effect of cation concentration on the formation and dissociation rates of dT:dA:dT triplex.** The association rate k_a (○) and dissociation rate k_d (●) were determined at different concentrations of Mg^{2+} ion. The analysis was made according to the Eq. 1. The association and dissociation were measured at 20 and 30°C, respectively.

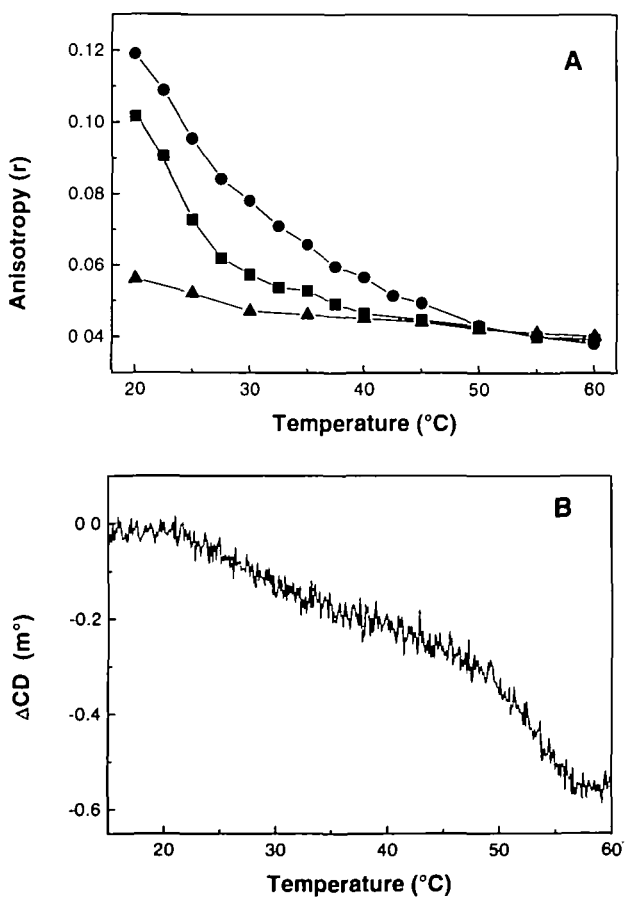

Fig. 4. **Thermal denaturation of triplex: dissociation of first and third (dT) strands from triplex.** The (dT):(dA):(dT) triplex (10 μM in base triplet) was formed by either adding fluorescein-labeled oligo(dT) to (dA):(dT) duplex (■) or adding non labeled (dT) to labeled duplex (●) at 2 mM $MgCl_2$. In panel A the dissociation of the labeled strand with temperature increase was monitored from the decrease of fluorescence anisotropy. The anisotropy of free oligo(dT) is also shown (▲). In panel B the denaturation was monitored by CD signal at 260 nm.

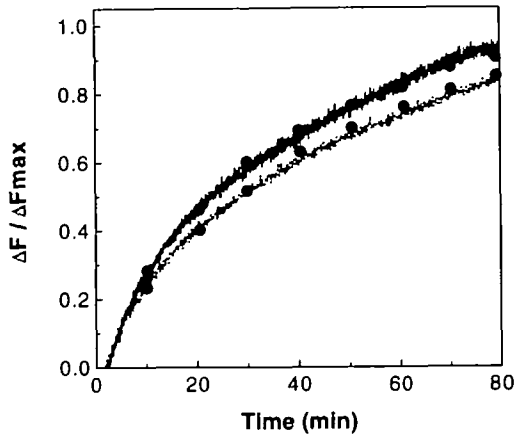

Fig. 5. **Dissociation kinetics of the third strand from triplex.** The dissociation of the fluorescein-labeled third dT strand from the triplex (3 μM in bases) was followed by fluorescence change upon the addition of 50-fold excess of non-labeled oligo(dT) (150 μM in bases). The experiments were performed at 30°C in the presence of 5 (dotted line) and 10 (continuous line) mM Mg^{2+}. The fluorescence intensity was normalized to that of final value which is close to that of free oligo(dT*) fluorescence. The best fit theoretical curves are also shown (•••).

labeled (dT) strand to the duplex formed with the labeled oligonucleotide (dA:dT*) or adding labeled (dT*) strand to non-labeled (dA:dT) duplex.

The dissociation of each strand was then investigated by change in anisotropy value upon the temperature increase and compared with the denaturation of the triplex followed by CD measurements (Fig. 4). For the both complexes, the fluorescence anisotropy value largely decreased with increase of temperature from 20 to 30°C. This change may be related to triplex-to-duplex transition. At 30°C the anisotropy of the third strand achieved to the value almost that of free oligo(dT*) indicating a complete dissociation while that of the first strand remained significantly larger. CD signal also indicates the first transition around this temperature. This confirms the model in which the dissociation of Hoogsteen bound third strand occurs at first and produces duplex DNA before the dissociation of Watson-Crick duplex.

By contrast, we could not detect the denaturation of duplex by fluorescence anisotropy measurement. The fluorescence anisotropy of the first strand progressively decreased with temperature arise around 40°C, where no significant transition was indicated by CD measurement, and achieved to the value of free oligo(dT*) at 50°C while the CD measurements showed that duplex-to-simplex transition occurs at 52°C. This may be related to the increase in mobility of the oligonucleotide with increase of temperature (*18*) without denaturation.

Dissociation Kinetics—The dissociation kinetics of the Hoogsteen-bound third strand from the triplex was analyzed by chase technique at 30°C, below melting temperature. Fifty-fold excess of non-labeled oligo(dT) was added to the triplex, which was formed by addition of the labeled dT* strand to dT:dA duplex. Because of large excess of non-labeled dT strand, the reassociation of dT* strand is negligible and one can thus follow the dissociation of dT* strand. The experiments were performed at 5 and 10 mM

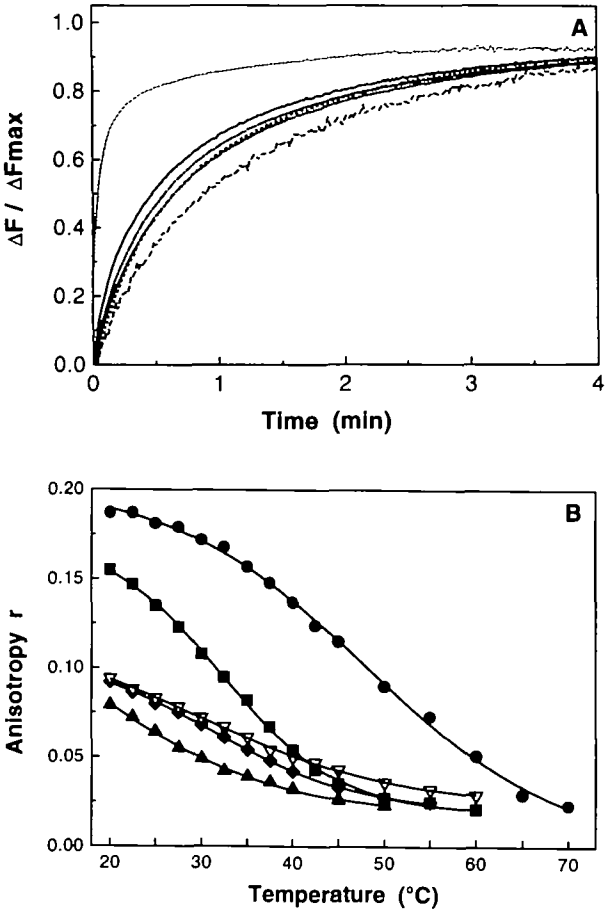

Fig. 6. **Effect of various divalent ions on the formation kinetics and stability of dT:dA:dT triplex.** In panel A, the kinetics of triplex formation was followed as described in Fig. 2A in the presence of 5 mM Mn^{2+} (·····), Mg^{2+} (——), Co^{2+} (—·—), Ni^{2+} (— —), Ca^{2+} (- - -), or Ba^{2+} (— —). In panel B, the denaturation of the triplex formed above upon the temperature elevation was monitored by the change in anisotropy of fluorescein-labeled dT strand. Mn^{2+} (●), Mg^{2+} (■), Ni^{2+} (▽), Ca^{2+} (◆), or Ba^{2+} (▲).

Mg^{2+} concentrations. In both cases the fluorescence intensity increased with time and achieved almost the value of uncomplexed oligo(dT*) showing the dissociation of the third strand from the triplex (Fig. 5). By contrast, no significant fluorescence change with time was observed when oligo(dT) was not added or the first strand of the triplex was labeled (not shown). The fluorescence change can be fitted with a first order mechanism (Fig. 5). The dissociation rate was found almost independent of Mg^{2+} concentration (2.0 and 1.8×10^{-2} min^{-1} at 5 and 10 mM Mg^{2+}, respectively) contrast to the association rate (Fig. 3).

Effect of Other Metal Ions—The effect of various metal ions on the stability of triplex DNA was reported. It depends upon the nature of metal ion and the sequence of triplex (*23, 24*). We have examined the effect of various divalent metal ions on the formation and the stability of dT:dA:dT triplex. The association rate was very different upon the nature of metal ions at a given ion concentration (5 mM). The formation rate of the triplex was faster in the order of $Mn^{2+}>Mg^{2+}>Ni^{2+}$, $Ca^{2+}>Ba^{2+}$ (Fig. 6A). With Ni^{2+}, Ca^{2+}, and Ba^{2+}, the fluorescence change was small and the reaction could be incomplete. In fact, the measurement

of fluorescence anisotropy change with elevation of temperature indicates instability of the triplex formed with these metal ions (Fig. 6B). In contrast to these ions, Mn^{2+} ion, which is the most efficient ion for the triplex formation, strongly stabilizes the triplex. There is a correlation between the stability and the formation rate of the triplex. By contrast, the dissociation rate was independent of nature of ions. The rate was found to be 1.8, 1.9, and 1.6×10^{-2} min^{-1} with Mn^{2+}, Mg^{2+}, and Ca^{2+}, respectively.

The strong effect of Mn^{2+} ion on the triplex formation could be due to higher intrinsic efficiency of this ion compared to the other ions. An alternative possibility is that the affinity of Mn^{2+} to the triplex is higher and rather low concentration (5 mM) is sufficient for the maximum effect while Mg^{2+} and other ions require much higher concentration to achieve the maximum effect, and thus the effect of Mn^{2+} appears stronger than that of Mg^{2+} at 5 mM. We have examined the effect of concentration of Mn^{2+} on the association rate. The reaction strongly depended upon the concentration and faster at higher ion concentration (not shown) just as like the case of Mg^{2+}. We could not discriminate either of explanations. The dissociation rate appeared independent of Mn^{2+} concentration from 2 to 10 mM (data not shown).

DISCUSSION

We have made use of fluorescein-labeled DNA for kinetic analysis of the triple helix formation and dissociation. The labeling technique offers unique possibility to follow directly the dissociation kinetics at below melting temperature as well as selective monitoring of the state of either of the strands. Furthermore, the fluorescence measurements are much more sensitive than CD or absorption measurements, and allow the detection in a wider range of DNA concentrations. The kinetic analysis can be easily performed at dilute polynucleotide conditions at which the rates could be readily followed in real time after mixing by hand. Exploiting these advantages, we were able to make the following conclusions: (1) metal ions affect positively the formation rate but not significantly the dissociation rate; (2) the effect of metal ions depends upon the nature and the concentration of ions.

The stabilization of triplex DNA by metal ions is well established (*4, 16, 17, 22-24*). We have here demonstrated that the metal ions affect the association rate but not the dissociation rate, and that the effect strongly depends upon their concentration. Probably only the DNA strand complexed by metal ions can interact with duplex DNA and form the triplex. This effect of metal ion can not be explained by counter ion shielding effects in contrast to the case of stabilization of duplex DNA. Pur:Pyr:Pur type triplex DNA is not stabilized by monovalent ion such as Na^{+}. The stabilization depends upon the nature of ions. The fact that the efficiency of divalent ions depends also upon the sequence of the triplex (*23, 24*) suggests that metal ions are directly involved in the base-base interaction in the triplex. The binding affinity of metal ions to these sites in the DNA may be weak and thus high concentration of ion is required for the stimulation effect.

Rougée and colleagues reported that the association rate is negatively influenced by concentration of monovalent ion (*15*). Monovalent ion competes with metal ion and chase it. At higher monovalent ion concentration, smaller part of DNA may be complexed by metal ion and the association rate may become slower. Their observation is thus compatible with our conclusion. They observed that the dissociation rate is not influenced by monovalent ion concentration (*15*). This is also in accord with our observation that the dissociation rate is almost independent of metal ion concentration.

No significant effect of Mg^{2+} and Mn^{2+} ions on the dissociation rate of the triplex indicate that the thermodynamic stabilization can be direct consequence of the increased reaction rate. The correlation between the efficiency of ions for the triplex stabilization and the acceleration of triplex formation further supports this conclusion. The dissociation rate of T:A:T triplex determined in this report (3.3×10^{-4} s^{-1}) is in the same order as that determined by Rougée and colleagues for another oligonucleotide sequence. It is interesting to examine the effect of sequence on the dissociation rate as well as the association rate.

The stabilization effect of Mn^{2+} and Mg^{2+} ions on triplex DNA strongly depends upon the ion concentration and is very large at high ion concentration. Extreme stability of RecA-promoted triplex has been reported and found remarkable (*1-4*). However, our observation showed that the conventional dT:dA:dT triplex is also very stable at the high Mg^{2+} concentrations (10 mM) which was used for the analysis of RecA-promoted triplex DNA. It is not obvious how much the latter is more stable than the conventional triplex.

In conclusion we have established a convenient fluorescence method, including chase technique, for kinetic analysis of formation and dissociation of triplex DNA and gained additional information about the mechanism of triplex formation.

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