Triplex Formation of Chemically Modified Homopyrimidine Oligonucleotides: Thermodynamic and Kinetic Studies†

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ABSTRACT: We have investigated effects of chemical modifications of a third strand on the thermodynamic and kinetic properties of the triplex formation between a 23-bp duplex and each of four kinds of 15-mer chemically modified third strands using isothermal titration calorimetry and interaction analysis system. The chemical modifications of the third strand included one base modification, with replacement of thymine by uracil; two sugar moiety modifications, RNA and 2′-*O*-methyl-RNA; and one phosphate backbone modification, with replacement of phosphodiester by phosphorothioate backbone. The thermodynamic and kinetic parameters obtained were similar in magnitude at room temperature for the triplex formation with the base-modified and the sugar-modified third strands. By contrast, binding constant for the triplex formation with the third strand containing phosphorothioate backbone was much smaller by a factor of 10 than that for the other triplex formations. Kinetic analyses have also demonstrated that the third strand containing phosphorothioate backbone was much slower in the association step and much faster in the dissociation step than the other third strands, which resulted in the much smaller binding constant. The reason for the instability of the triplex with the third strand containing phosphorothioate backbone will be discussed. We conclude that, at least in the triplex formation with the chemically modified third strands studied in the present work, the modification of phosphate backbone of the third strand produces more significant effect on the triplex formation than the modifications of base and sugar moiety.

In recent years, triplex DNA has attracted considerable interest because of its possible biological function *in vivo* and its wide variety of potential applications, such as regulation of gene expression, site-specific cleavage of DNA, and mapping of genomic DNA (*1*−*3*). Triplex is usually formed through sequence-specific interaction between a homopurine−homopyrimidine stretch of duplex and a homopyrimidine or homopurine single strand (*1*−*3*). In the pyrimidine-motif triplex formation, a third homopyrimidine single strand binds to the major groove of the duplex in parallel with the homopurine strand via Hoogsteen hydrogen bonding (*1*−*3*). Stable base triplets in the pyrimidine-motif triplex, T·AT and C^+·GC, are formed between thymines (T) in the third strand and adenine·thymine (AT) base pairs of duplex and between protonated cytosines (C^+) in the third strand and guanine·cytosine (GC) base pairs of duplex, respectively (*1*−*3*). In the purine-motif triplex formation, a third homopurine single strand binds to the major groove of the duplex antiparallel to the other homopurine strand via reverse Hoogsteen hydrogen bonds (*1*−*3*). A·AT, T·AT, and G·GC are stable base combinations in the purine-motif triplex (*1*−*3*). The stability of triplex is affected by various environmental conditions, such as pH, temperature, ionic strength, and supercoil density (*4*−*7*).

Various kinds of chemical modifications in base, sugar moiety, and phosphate backbone have been developed to enhance the binding affinity and modify the sequence specificity of triplex formation (*3*, *8*, *9*). To overcome the requirement of acidic pH for the pyrimidine-motif triplex formation and stabilize the pyrimidine-motif triplex at neutral pH, several base analogues have been synthesized, such as 5-methylcytosine (*10*−*15*), N^6-methyl-8-oxo-2′-deoxyadenosine (*16*, *17*), 2′-*O*-methylpseudoisocytidine (*18*, *19*), and 1-(2-deoxy-β-D-ribofuranosyl)-3-methyl-5-amino-1*H*-pyrazolo[4,3-*d*]pyrimidin-7-one (*20*, *21*). Modifications in sugar moiety have also been developed. RNA is the most obvious example of substitution on the deoxyribose sugar moiety (*22*−*30*). To avoid degradation of RNA by nuclease, 2′-*O*-methyl-RNA has been designed, in which the 2′ position of the sugar moiety is replaced with a methoxy group (*23*, *29*, *31*−*33*). To change the electrostatic properties of the negative phosphodiester backbone of natural DNA molecules and achieve greater degrees of nuclease resistance and cell membrane permeability, several backbone modifications have been designed, such as phosphorothioate (*34*−*38*) and phosphoramidate (*39*−*43*) backbones. Peptide nucleic acids with polyamide backbones have been popular for the past few years (*44*−*48*).

Although the denaturation process of triplex containing a chemically modified homopyrimidine third strand far above physiological temperature has been investigated by UV

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melting (*10*, *12*, *15*, *18*, *19*, *22*−*24*, *28*−*31*, *33*, *34*, *36*, *38*, *42*) and differential scanning calorimetry (DSC)[1] (*12*, *15*), the formation process of triplex with a chemically modified third strand near physiological temperature has not been well characterized yet. To apply triplex as an antigene drug for artificial control of gene expression *in vivo*, the investigation of the formation process of triplex near physiological temperature is much more important than that of the dissociation process far above physiological temperature. In addition, some controversy has arisen among the previously reported results concerning the effect of chemical modifications of a third strand on the stability of triplex. For example, Han and Dervan (*25*) and Gotfredsen *et al*. (*30*) showed that a homopyrimidine DNA third strand and the corresponding RNA third strand bound to the target duplex DNA with almost the same binding constant. On the other hand, Roberts and Crothers (*22*), Escude *et al*. (*23*, *24*), and Liquier *et al*. (*28*) reported that the triplex with an RNA third strand was significantly more thermally stable than that with the corresponding DNA third strand. Furthermore, we have previously demonstrated that triplex formation near room temperature (15−35 °C) is not expressed by a simple two-state model but exhibits multiple states, and we have also suggested that a self-structure of a third strand may affect the thermodynamics of triplex formation (*7*). A more detailed mechanism remains to be established.

In the present study, we have further extended our study to explore the effect of chemical modifications of a third strand on the thermodynamic and kinetic properties of triplex formation at room temperature. The thermodynamic and kinetic properties have been analyzed by isothermal titration calorimetry (ITC) (*7*, *49*−*52*) and interaction analysis system (IAsys) (*52*−*55*), respectively. The chemical modifications of a third strand included one base modification, two sugar moiety modifications, and one phosphate backbone modification. Few studies have been reported to compare the thermodynamic and kinetic properties of the triplex formation with the same target duplex DNA among such various kinds of chemically modified third strands. Comparing with the results for the unmodified third strand, we have found that, at least in triplex formation with the chemically modified third strands studied in the present work, the modification of phosphate backbone of the third strand has produced a more significant effect on the thermodynamics and kinetics of the triplex formation than the modifications of base and sugar moiety.

MATERIALS AND METHODS

Preparation of Oligonucleotides. We synthesized a 15-mer DNA oligonucleotide, 5′-CTCTTCTTTTCTTTC-3′ (denoted as Pyr15T), and complementary 23-mer DNA oligonucleotides, 5′-GCGCGAGAAGAAAAG AAAGCCGG-3′ (denoted as Pur23A) and 5′-CCGGCTTTCTTTTCTTCTCG-CGC-3′ (denoted as Pyr23T), on an ABI 381A DNA synthesizer, by a solid-phase cyanoethyl phosphoramidite method, and purified them by a reverse-phase HPLC on a Wakosil DNA column. 5′-Biotinylated Pyr23T (Bt-Pyr23T) was prepared from biotin phosphoramidite. The concentration of the single-stranded oligonucleotides was determined by UV absorbance (*56*, *57*). The complementary strands, Pur23A and Pyr23T, were annealed by heating to 90 °C, followed by gradual cooling to room temperature. The annealed sample was passed through a hydroxyapatite column (Koken) to remove any unpaired single strands. The concentration of the 23-bp double-stranded DNA, Pur23A·Pyr23T, was determined by UV absorption with a DNA concentration ratio of 1 OD = 50 μg/mL. We also prepared four chemically modified oligonucleotides as the third strand: Pyr15dU, 15-mer DNA where T of Pyr15T is replaced with U; Pyr15RNA, 15-mer RNA with the sequence corresponding to Pyr15T; Pyr15OMe, 15-mer RNA with the same sequence as Pyr15RNA but with 2′-*O*-methylated riboses; and Pyr15P, 15-mer DNA with the same sequence as Pyr15T but with a phosphorothioate backbone. The replacement of a nonbridging oxygen by a sulfur atom in a phosphodiester group produces two stereoisomers. Since the automated synthesis of DNA cannot be stereospecific, Pyr15P is a mixture of diastereomers. The purified oligonucleotide solutions were dialyzed extensively against the experimental buffer.

Isothermal Titration Calorimetry. Isothermal titration experiments were carried out on a MCS ITC system (Microcal Inc., USA) interfaced with a microcomputer (*7*, *49*−*52*). The Pur23A·Pyr23T duplex DNA and the third-strand oligonucleotide solutions were prepared by extensive dialysis against the experimental buffer (10 mM sodium cacodylate, 10 mM acetic acid, 200 mM sodium chloride, and 20 mM magnesium chloride, pH 4.9). The third-strand oligonucleotide was injected 20 times in 5 μL increments and 5 min intervals into the Pur23A·Pyr23T duplex DNA solution. The heat for each injection was subtracted by the heat of dilution of the injectant, the latter of which was measured by injecting the third-strand oligonucleotide into the dialysis buffer without duplex DNA. Each corrected heat was divided by the moles of the third-strand oligonucleotide injected and analyzed with Microcal Origin software supplied by the manufacturer.

Interaction Analysis System. Kinetic experiments were performed on an IAsys instrument (Affinity Sensors, Cambridge, U.K.) interfaced with a microcomputer, where a real-time biomolecular interaction was measured with a laser biosensor (*52*−*55*). The carboxymethylated dextran resonant layer of a cuvette was washed with 200 μL of 10 mM acetate buffer at pH 4.6, and then activated with 200 μL of a mixture of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and *N*-hydroxysuccinimide solution. The activated surface was washed with 10 mM acetate buffer at pH 4.6, and streptavidins were immobilized to the surface. After the remaining reactive groups were blocked with 1 M ethanolamine at pH 8.5 and the surface was washed with 10 mM acetate buffer at pH 4.6, loosely associated proteins were removed with 20 mM hydrochloric acid washes. After the cuvette was washed with the hybridization buffer (10 mM sodium cacodylate/10 mM acetic acid at pH 4.9, containing 200 mM sodium chloride and 20 mM magnesium chloride), Bt-Pyr23T (1.2 μM in the hybridization buffer) was captured

[1] Abbreviations: DSC, differential scanning calorimetry; ITC, isothermal titration calorimetry; IAsys, interaction analysis system; Pyr15T, 15-mer DNA as unmodified homopyrimidine third strand; Pur23A·Pyr23T, 23-bp target duplex DNA; Bt-Pyr23T, biotinylated Pyr23T; Pyr15dU, 15-mer DNA where T of Pyr15T is replaced with U; Pyr15RNA, 15-mer RNA with the sequence corresponding to Pyr15T; Pyr15OMe, 15-mer RNA with the same sequence as Pyr15RNA but with 2′-*O*-methylated riboses; Pyr15P, 15-mer DNA with the same sequence as Pyr15T but with phosphorothioate backbone.

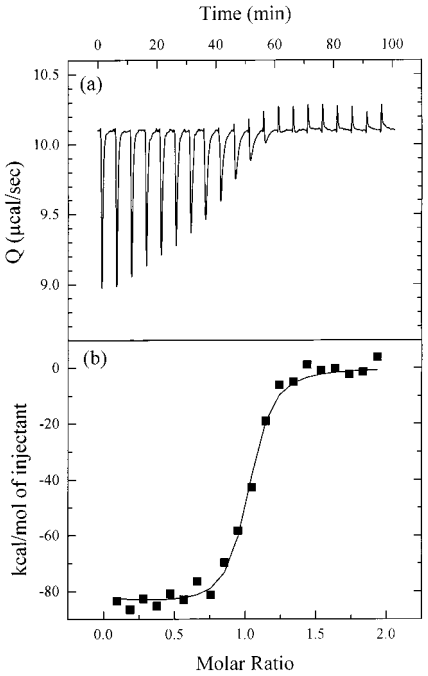

FIGURE 1: Typical isothermal titration calorimetric profiles of the triplex formation between Pyr15dU and Pur23A·Pyr23T at 25 °C and pH 4.9. (a) The Pyr15dU solution (120 μM in 10 mM sodium cacodylate, 10 mM acetic acid, 200 mM sodium chloride, and 20 mM magnesium chloride, pH 4.9) was injected 20 times in 5 μL increments into 5 μM Pur23A·Pyr23T solution, which was dialyzed against the same buffer. Injections were made for 10 s at 5 min intervals. (b) Integrated areas for the above peaks were plotted against the molar ratio of [Pyr15dU]/[Pur23A·Pyr23T]. The data were fitted by use of a nonlinear least-squares method.

to the streptavidin on the surface. The cuvette was washed with the hybridization buffer, and the complementary oligonucleotide, Pur23A (1.2 μM in the hybridization buffer), was added to hybridize with Bt-Pyr23T on the surface. After washing and equilibration of the immobilized surface with the experimental buffer for more than 30 min, Pyr15T or the 15-mer chemically modified homopyrimidine third strand was injected over the immobilized Bt-Pyr23T·Pur23A duplex in the experimental buffer, and then the triplex formation was monitored for 30 min. After replacing with the experimental buffer, the dissociation of the complex was monitored for 15 min. Sodium hydroxide solution (10 mM) was injected for 3 min to dissociate the third strand from the target duplex completely, when the Bt-Pyr23T·Pur23A duplex may be partially denatured. Regeneration of the immobilized Bt-Pyr23T·Pur23A duplex was carried out by injection of 1.2 μM Pur23A. The resulting sensorgrams were analyzed with the Fastfit software supplied by the manufacturer to calculate the kinetic parameters.

RESULTS

Determination of Thermodynamic Parameters of Triplex Formation by ITC. We have examined thermodynamics of the triplex formation between a 23-bp double-stranded DNA, Pur23A·Pyr23T, and a 15-mer unmodified single-stranded homopyrimidine DNA, Pyr15T, or each of four kinds of 15-mer chemically modified single strands, Pyr15dU, Pyr15RNA, Pyr15OMe, and Pyr15P, by ITC. Figure 1a shows a typical ITC profile for the interaction between Pyr15dU and Pur23A·Pyr23T at 25 °C and pH 4.9. An exothermic heat pulse was observed after injection of Pyr15dU into Pur23A·Pyr23T in the experimental buffer. The magnitude of each peak decreased gradually with successive injections, and a small endothermic peak was still observed at a molar ratio of [Pyr15dU]/[Pur23A·Pyr23T] = 2. The endothermic peak was proved to be the heat of dilution of Pyr15dU by a blank experiment in the absence of Pur23A·Pyr23T. The area under each peak was integrated, and the heat of dilution of Pyr15dU was subtracted from the integrated values. The corrected heat was divided by the moles of each injection, and the resulting values were plotted as a function of molar ratio of [Pyr15dU]/[Pur23A·Pyr23T], as shown in Figure 1b. The resultant titration plot was well fitted to a sigmoidal curve by a nonlinear least-squares method. The binding constant, K_a, and the enthalpy change, ΔH, were obtained from the fitted curve. Further, the Gibbs free energy change, ΔG, and the entropy change, ΔS, were calculated from the equation $\Delta G = -RT \ln K_a = \Delta H - T\Delta S$. At least three experiments were carried out and the obtained parameters were averaged. The thermodynamic parameters of the triplex formation with Pyr15T or the other chemically modified third strands, Pyr15RNA, Pyr15OMe, and Pyr15P, were obtained in the same way and summarized in Table 1.

As can be seen in Table 1, both ΔH and ΔS are largely negative in all cases, indicating that the triplex formation with each of the oligonucleotides used here is driven by a large negative ΔH. K_a was in the order of 10^7 M^{-1} for the association reaction of Pur23A·Pyr23T with each of Pyr15T, Pyr15dU, Pyr15RNA, and Pyr15OMe. By contrast, K_a for the association with Pyr15P was around 10^6 M^{-1}, which was less than $^1/_{10}$ of that for the other triplex formations. The magnitudes of the negative ΔH and ΔS for the association with Pyr15P were also 2−3 times and 2.4−3.6 times smaller than those for the other associations, respectively. The above results indicate that the modification of phosphate backbone resulted in dramatic effects on the thermodynamic parameters for the triplex formation, whereas the modifications of base and sugar moiety gave little effect.

Determination of Kinetic Parameters of Triplex Formation by IAsys. To obtain more information about the triplex formation with the chemically modified oligonucleotides, we have carried out kinetic experiments by IAsys. Figure 2a shows a typical IAsys sensorgram representing triplex formation between Pyr15RNA and Pur23A·Pyr23T at 25 °C and pH 4.9. An injection of Pyr15RNA over the Bt-Pyr23T·Pur23A duplex immobilized to a biosensor tip caused an increase in response, corresponding to triplex formation. We measured a series of association curves for 30 min and dissociation curves for 15 min after injections of various concentrations of Pyr15RNA. The on-rate constants, k_{on}, were obtained from the analysis of the association curves. Figure 2b shows a plot of k_{on} against the concentrations of Pyr15RNA. The resultant plot was fitted to a straight line by a linear least-squares method. The association rate constant, k_{assoc}, and the dissociation rate constant, k_{dissoc}, were determined from the slope and the intercept of the regression line, respectively. K_a was calculated from the equation $K_a = k_{assoc}/k_{dissoc}$. Since the change of the dissociation curves with time was very small for all kinds of third strands (Figure 2a), that is, the dissociation was very slow, we could not directly determine the dissociation rate constant, k_{dissoc}, from

Table 1: Thermodynamic Parameters[a] for Triplex Formation with a Series of Chemically Modified Homopyrimidine Single Strands

single strand	K_a (M^{-1})	ΔG (kcal mol^{-1})	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} K^{-1})
Pyr15T	$(2.76 \pm 0.34) \times 10^7$	-10.2 ± 0.08	-81.5 ± 1.3	-239 ± 4.6
Pyr15dU	$(3.16 \pm 0.53) \times 10^7$	-10.2 ± 0.11	-78.7 ± 3.7	-230 ± 12.8
Pyr15RNA	$(2.62 \pm 0.015) \times 10^7$	-10.1 ± 0.01	-67.8 ± 0.75	-193 ± 2.5
Pyr15OMe	$(2.77 \pm 0.34) \times 10^7$	-10.2 ± 0.08	-58.3 ± 2.6	-161 ± 9.0
Pyr15P	$(1.66 \pm 0.43) \times 10^6$	-8.48 ± 0.18	-29.0 ± 1.4	-68.8 ± 5.3

[a] Obtained by ITC measurements at 25 °C and pH 4.9. Concentration of the 15-mer single strand except Pyr15P was 120 μM in the syringe. Concentration of Pyr15P was 240 μM in the syringe. The single strand was injected 20 times in 5 μL increments into the duplex. Concentration of the 23-bp duplex for the injection of the single strand except Pyr15P was 5 μM in the cell. Concentration of the duplex for the injection of Pyr15P was 10 μM in the cell. The obtained values are the average of at least three ITC experiments.

Table 2: Kinetic Parameters[a] for Triplex Formation with a Series of Chemically Modified Homopyrimidine Single Strands

single strand	k_{assoc} (M^{-1} s^{-1})	k_{dissoc} (s^{-1})	K_a (M^{-1})
Pyr15T	$(1.05 \pm 0.021) \times 10^4$	$(5.07 \pm 0.97) \times 10^{-4}$	$(2.07 \pm 0.37) \times 10^7$
Pyr15dU	$(1.83 \pm 0.13) \times 10^4$	$(6.15 \pm 7.8) \times 10^{-4}$	$(2.98 \pm 1.76) \times 10^7$
Pyr15RNA	$(1.36 \pm 0.044) \times 10^4$	$(4.88 \pm 2.3) \times 10^{-4}$	$(2.79 \pm 0.95) \times 10^7$
Pyr15OMe	$(7.92 \pm 0.59) \times 10^3$	$(2.77 \pm 3.1) \times 10^{-4}$	$(2.86 \pm 1.61) \times 10^7$
Pyr15P	$(2.46 \pm 0.55) \times 10^3$	$(4.87 \pm 0.45) \times 10^{-3}$	$(5.05 \pm 1.46) \times 10^5$

[a] Obtained by IAsys measurements at 25 °C and pH 4.9. Several different concentrations of the 15-mer single strand were injected over the immobilized 23-bp duplex. The concentrations of the injected single strand were between 0.1 and 2 μM.

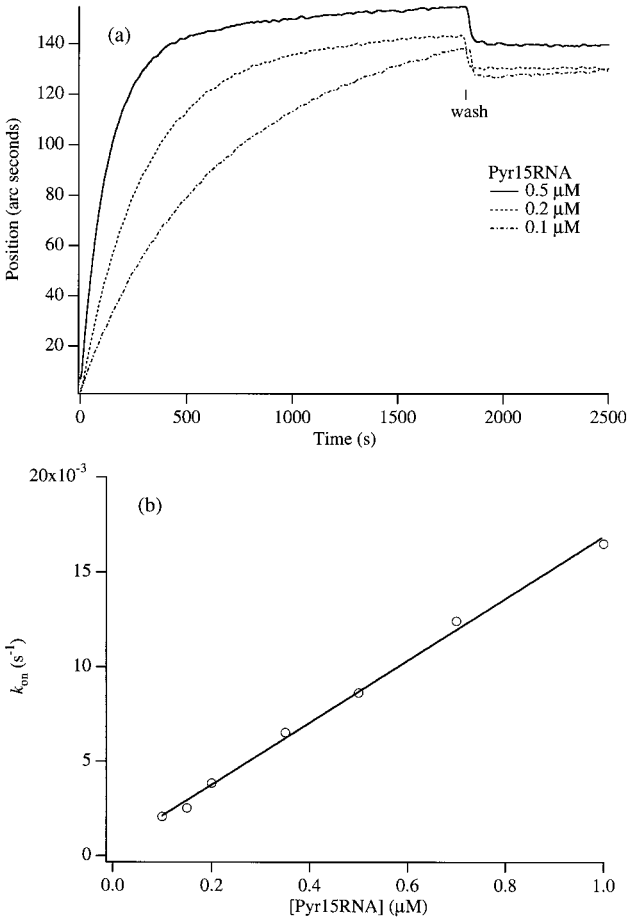

FIGURE 2: Typical interaction analysis system sensorgram of the triplex formation between Pyr15RNA and Pur23A·Pyr23T at 25 °C and pH 4.9. (a) Portions (200 μL) of the serially diluted Pyr15RNA solutions in 10 mM sodium cacodylate, 10 mM acetic acid at pH 4.9 containing 200 mM sodium chloride and 20 mM magnesium chloride were poured into Bt-Pyr23T·Pur23A-immobilized cuvette. The binding of Pyr15RNA to Bt-Pyr23T·Pur23A was monitored as the resonance position against time. (b) Measured on-rate constants, k_{on}, for the different concentrations of Pyr15RNA were plotted against their respective concentrations. The plot was fitted to a straight line by use of a linear least-squares method.

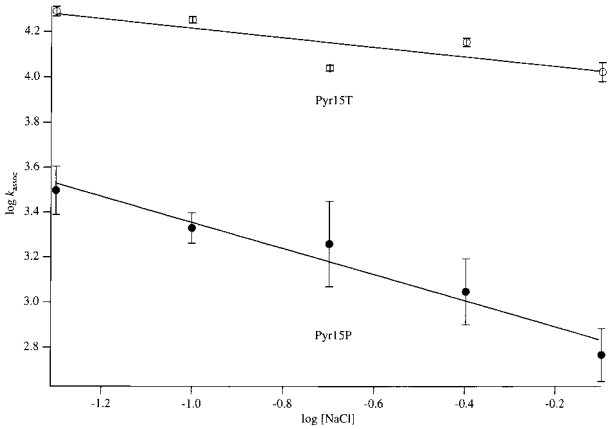

FIGURE 3: Dependence of the log k_{assoc} values of Pyr15T and Pyr15P for Pur23A·Pyr23T upon the logarithm of the sodium chloride concentration at 25 °C. All experiments were performed in 10 mM sodium cacodylate, 10 mM acetic acid at pH 4.9 containing 20 mM magnesium chloride and the experimental concentration of sodium chloride.

the dissociation curves. Thus, k_{dissoc} obtained from the association curves was presented, although its standard deviation was relatively large (Table 2). The kinetic parameters for the triplex formation with Pyr15T or the other chemically modified third strands, Pyr15dU, Pyr15OMe, and Pyr15P, were obtained in the same way and summarized in Table 2.

As can be seen in Table 2, for triplex formation with each of Pyr15T, Pyr15dU, Pyr15RNA, and Pyr15OMe, the association rate constants, k_{assoc}, fall in a narrow range between 8×10^3 and 2×10^4 M^{-1} s^{-1}, and the dissociation rate constants, k_{dissoc}, are in the order of 10^{-4} s^{-1}. By contrast, the k_{assoc} and k_{dissoc} for Pyr15P are 2.5×10^3 M^{-1} s^{-1} and 4.9×10^{-3} s^{-1}, respectively, which are quite different from the others. The K_a values calculated from the ratio of k_{assoc} to k_{dissoc} were in good agreement with those obtained from ITC for the triplex formation with all kinds of third strands (Table 1), indicating reliability of K_a obtained by the two different methods. Clearly, the K_a value for the triplex formation with Pyr15P was less than $^1/_{10}$ of that for the other

triplex formations, which was also consistent with the results of ITC. Thus, the low binding affinity of Pyr15P to Pur23A·Pyr23T was ascribed to both small k_{assoc} and large k_{dissoc} values.

Salt Concentration Dependence of the Association Rate Constants of the Triplex Formation with Pyr15T and Pyr15P. To understand the small k_{assoc} observed for Pyr15P, the k_{assoc} between Pur23A·Pyr23T and each of Pyr15T and Pyr15P were measured by IAsys as a function of salt concentration in the range of 50−800 mM sodium chloride at 25 °C and pH 4.9. As the sodium chloride concentration was increased in the presence of 20 mM magnesium chloride, the logarithms of k_{assoc} for Pyr15T and Pyr15P decreased almost linearly with respect to log [NaCl] as shown in Figure 3. The slopes of the regression lines in Figure 3 were -0.21 ± 0.08 and -0.58 ± 0.07 for Pyr15T and Pyr15P, respectively. Thus, the slope for Pyr15P was nearly 3 times larger than that for Pyr15T. Since the standard deviation of k_{dissoc} determined from the association curves was relatively large, we could not obtain reproducible data on the salt concentration dependence of k_{dissoc}.

DISCUSSION

No significant differences in the thermodynamic and kinetic parameters were observed between triplex formation with Pyr15T and Pyr15dU (Tables 1 and 2). These results show that the methyl group in the pyrimidine ring virtually gives no significant effect on the triplex formation. Although Povsic and Dervan (*11*) reported that the triplex with a third strand containing deoxyuridine is less stable than the corresponding triplex with a third strand containing deoxythymidine, the effect of the methyl group in the pyrimidine ring on the stability of triplex was small. We believe that only a small, if even any, contribution to the stability of triplex may be expected from the methyl group in pyrimidine ring at pH 4.9.

The K_a value and the kinetic parameters of the triplex formation with Pyr15RNA were almost the same as those with Pyr15T within experimental error (Tables 1 and 2). Our data of the thermodynamic stability support the previously reported results by Han and Dervan (*25*) and Gotfredsen *et al*. (*30*). Gotfredsen *et al*. (*30*) showed that the three-dimensional NMR structure of the triplex containing an 8-mer RNA third strand at pH 5.7 in the presence of 100 mM sodium chloride more closely resembled the conformation of B-DNA rather than A-DNA, and the thermodynamic stability of the triplex with the RNA third strand was almost the same as that with the corresponding DNA third strand. On the other hand, Roberts and Crothers (*22*), Escude *et al*. (*23*, *24*), and Liquier *et al*. (*28*) showed that the triplex with an RNA third strand was significantly more stable than that with the corresponding DNA third strand. Liquier *et al*. (*28*) also reported an FT-IR study showing that the triplex containing a 7-mer RNA third strand at pH 4.8 in the presence of 20 mM sodium chloride with significantly higher thermal stability adopted mainly the A-DNA conformation. These results suggest that thermodynamic stability of a B-DNA-like triplex with an RNA third strand is almost the same as that with the corresponding DNA third strand, whereas a A-DNA-like triplex with an RNA third strand is significantly more stable than that with the corresponding DNA third strand. The triplex with Pyr15RNA in the present study may belong to the first category.

The magnitude of the negative ΔS for Pyr15RNA was smaller than that for Pyr15T (Table 1). We have previously suggested that the negative ΔS measured by ITC is mainly contributed by a negative conformational entropy change due to the conformational restraint of the third strand upon the triplex formation (*7*). An RNA single strand in the free state is usually more rigid than the corresponding DNA single strand (*58, 59*). Thus, the magnitude of a negative conformational entropy change for Pyr15RNA upon triplex formation should be smaller than that for Pyr15T, which may result in the smaller magnitude of the negative ΔS observed for Pyr15RNA.

The magnitude of the negative ΔH for Pyr15RNA was also smaller than that for Pyr15T (Table 1). The favorable contribution to the K_a and ΔG of the triplex formation by the smaller magnitude of the negative ΔS for Pyr15RNA was almost canceled out by the smaller magnitude of the negative ΔH (Table 1). The B-DNA-like conformation of the triplex with Pyr15RNA as discussed above may be one of the reason for the smaller magnitude of the negative ΔH. We conclude that the smaller magnitude of the negative ΔH for Pyr15RNA, probably due to a B-DNA-like conformation, may make it impossible to enhance the stability of the triplex with Pyr15RNA.

The K_a value of the triplex formation with Pyr15OMe was the same as that with Pyr15T within experimental error (Tables 1 and 2), which was inconsistent with the results obtained by other groups. Escude *et al*. (*23*), Shimizu *et al*. (*31*), and Dagneaux *et al*. (*33*) reported a UV melting study that the thermal stability of the triplex with a 2′-*O*-methyl-RNA third strand was significantly higher than that with the corresponding DNA third strand. The reason for the discrepancy between our data obtained from ITC and IAsys at room temperature and their results given by UV melting far above room temperature is unknown.

The magnitudes of both the negative ΔH and ΔS for Pyr15OMe were smaller than those for Pyr15T (Table 1), as in the case of Pyr15RNA. The smaller magnitude of the negative ΔS for Pyr15OMe may result from higher conformational rigidity of a 2′-*O*-methyl-RNA single strand in the free state than the corresponding DNA single strand (*7*, *60*, *61*). The favorable contribution to the K_a and ΔG of the triplex formation by the smaller magnitude of the negative ΔS for Pyr15OMe was almost canceled out by the smaller magnitude of the negative ΔH (Table 1). We conclude that the same K_a and ΔG of the triplex formation with Pyr15OMe relative to that with Pyr15T may result from the cancellation by the smaller magnitude of the negative ΔH.

Unlike the chemical modifications of base and sugar moiety discussed above, the K_a value of the triplex formation with Pyr15P was smaller by a factor of 10 than that with Pyr15T (Tables 1 and 2). Our data are consistent with the previously reported results (*36*−*38*). The magnitudes of both the negative ΔH and ΔS for Pyr15P were much smaller than those for Pyr15T (Table 1), indicating that the smaller K_a for Pyr15P is related to both enthalpic and entropic terms. The difference in thermodynamic parameters should be attributed to the difference in the following properties between Pyr15T and Pyr15P. First, since the van der Waals

radius of sulfur is larger than that of oxygen, the triplex with Pyr15P may be more sterically crowded than that with Pyr15T. The unfavorable steric effect may lead to the destabilization of the triplex formation with Pyr15P. Second, the charge distribution on the phosphorothioate backbone is different from that on the phosphodiester backbone (*62*, *63*). The previous calculation of the charge distribution in phosphate-related anions showed that negative charge was delocalized over the two nonbridging oxygens in PO_2 groups, whereas the sulfur bears greater negative charge than the oxygen in POS groups (*62*, *63*). Due to the difference in the charge distribution between phosphodiester and phosphorothioate backbones, the electrostatic repulsion between Pyr15P and Pur23A·Pyr23T should be different from that between Pyr15T and Pur23A·Pyr23T, which may lead to the change of the triplex stability. Third, the difference in the charge distribution between phosphodiester and phosphorothioate backbones may alter the distribution of coexisting cations around internucleotide linkages to stabilize the triplex formation.

To examine the third term as mentioned above, we have compared the salt concentration dependence of k_{assoc} of the triplex formation with Pyr15T and Pyr15P (Figure 3). As the sodium chloride concentration, [NaCl], was increased in the presence of 20 mM magnesium chloride, the logarithms of k_{assoc} for Pyr15T and Pyr15P decreased almost linearly with respect to log [NaCl] (Figure 3). Our data are similar to the previous study that the increase in [NaCl] at constant concentrations of magnesium chloride and spermine substantially decreased k_{assoc} and K_a of the triplex formation with the third strand containing 5-methylcytosine modification (*64*). The salt concentration dependence of k_{assoc} of the triplex formation may be related to at least the following factors: (1) the screening effect to minimize the repulsion of the anionic phosphate charges between the duplex and the third strand in the triplex formation and (2) the entropic effect to condense or release the coexisting cations on the DNA surface in the triplex formation. The first factor usually contributes to increase k_{assoc} upon the increase in [NaCl]. On the other hand, for the second factor, when coexisting cations are condensed on the DNA surface upon the increase in [NaCl], the sign of the entropic effect is negative, leading to a decrease in K_a and k_{assoc}, and the opposite case is also true. In our case the observed k_{assoc} decreased upon the increase in [NaCl] for both Pyr15T and Pyr15P (Figure 3), suggesting that coexisting cations may be condensed on the DNA surface in the triplex formation for both Pyr15T and Pyr15P upon the increase in [NaCl]. However, the dependence of the logarithm of k_{assoc} for Pyr15P on log[NaCl] was nearly 3 times larger than that for Pyr15T (Figure 3). We suggest that the amount of the condensation of coexisting cations upon the triplex formation may be different between the two triplexes and that Pyr15P may be entropically less favorable for the triplex formation than Pyr15T as far as the effect of salt concentration is concerned. Since the apparent negative ΔS observed by ITC is mainly contributed by a negative conformational entropy change due to the conformational restraint of the third strand upon the triplex formation rather than an entropy change due to the condensation of coexisting cations (*7*), the fact that the magnitude of the apparent negative ΔS upon the triplex formation with Pyr15P was smaller than that with Pyr15T (Table 1) suggests that phosphorothioate backbone in the free state might be more rigid than phosphodiester backbone.

In our previous ITC study, we have examined the mechanism of triplex formation near room temperature (*7*). To explain the observed large magnitude of heat capacity change, we had to consider the contributions of intermediate states during triplex formation and the conformational variety of the free third strand in addition to the solvation effect upon triplex formation. Thus, we have reached the conclusion that triplex formation is not expressed by a simple two-state model but exhibits multiple states, and a self-structure of the third strand may affect the thermodynamics of the triplex formation. On the other hand, the heat capacity change upon the denaturation process of the triplex in the previous DSC study by other groups (*12*, *15*) was zero, which was in sharp contrast with our ITC study of the triplex formation. The ITC experiment may provide useful information near physiological temperature, which was not directly obtained from the DSC experiment far above physiological temperature. Also, to apply triplex as an antigene drug for artificial control of gene expression *in vivo*, investigation of the triplex formation near physiological temperature is essential. Thus, in the present study we have employed ITC and IAsys near room temperature to examine thermodynamic and kinetic properties of the triplex formation between the 23-bp double strand and each of the 15-mer unmodified or four kinds of chemically modified third strands. The thermodynamic and kinetic parameters for triplex formation with the third strand containing a phosphorothioate backbone were quite different from those with the unmodified, base-modified, and sugar-modified third strands. In our previous study, we have suggested that conformational transition of the third strand along the phosphate backbone may affect the thermodynamics of triplex formation (*7*). Combining our previous and present studies (*7*), we suggest that, at least in triplex formation with the chemically modified third strands studied in the present work, chemical modifications of the phosphate backbone of the third strand are more effective to change the triplex stability than those of base and sugar moiety. This information will present an effective approach for designing chemically modified oligonucleotides with high binding affinity in triplex formation.

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