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Microcalorimetrics Studies of the Thermodynamics and Binding Mechanism between L-Tyrosinamide and Aptamer

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In recent years, several high-resolution structures of aptamer complexes have shed light on the binding mode and recognition principles of aptamer complexe interactions. In some cases, however, the aptamer complex binding behavior and mechanism are not clearly understood, especially with the absence of structural information. In this study, it was demonstrated that isothermal titration calorimetry (ITC) and circular dichroism (CD) were useful tools for studying the fundamental binding mechanism between a DNA aptamer and L-tyrosinamide (L-TyrNH_2). To gain further insight into this behavior, thermodynamic and conformational measurements under different parameters such as salt concentration, temperature, pH value, analogue of L-TyrNH_2, and metal ion were carried out. The thermodynamic signature along with the coupled CD spectral change suggest that this binding behavior is an enthalpy-driven process, and the aptamer has a conformational change from B-form to A-form. The results showed that the interaction is an induced fit binding, and the driving forces in this binding behavior may include electrostatic interactions, hydrophobic effects, hydrogen bonding, and the binding-linked protonation process. The amide group and phenolic hydroxyl group of the L-TyrNH_2 play a vital role in this binding mechanism. In addition, it should be noted that Mg^{2+} not only improves binding affinity but also helps change the structure of the DNA aptamer.

1. Introduction

Molecular recognition between nucleic acids and their target substrates is essential in many biological processes including transcription and translation. For instance, Tat protein-TAR RNA interaction with both HIV-1 and HIV-2 is critical for viral replication.[1] Recently, it was found that small functional RNA motifs have an important regulatory role in the control of gene expression. Important examples are riboswitches, which regulate RNA translation via the aptamer domain of mRNA binding to metabolites,[2] and RNAi, which suppresses gene expression by complementary base pairing.[3] Since Watson and Crick[4] constructed the DNA double helix model in 1953, the interaction of the nucleic acid has been studied extensively. However, the mechanism from the thermodynamics aspect of DNA−ligand binding is still not fully explained. The ligand-binding of riboswitches and base-pairing of RNAi are still phenomena in vivo. To understand these binding behaviors, an in vitro experimental design is therefore necessary and will prove informative.

In 1990, an important technique emerged, which uses in vitro selection approaches to isolate RNA and DNA molecules that bind nucleic acids binding protein.[5–7] The in vitro selection procedure is called "systemic evolution of ligands by exponential enrichment" (SELEX), and the products were named "aptamers". ssDNA and RNA aptamers selectively bind to their target amino acids,[8–10] peptides,[11] proteins,[12] nucleotides,[7] carbohydrates,[13] and other molecules.[14,15] The binding of aptamers and their ligand often rely on the secondary and tertiary structural elements of the nucleic acid. Some studies suggest that the in vitro selected aptamers structure is similar to that of the RNA in the bacterium's biosynthetic pathway,[16] indicating that such small functional RNA motifs may exist in cells.[17] However, the function and evolutionary mechanism of these RNAs are not clear.

The binding mechanism between the target ligand and its aptamers may be complicated and varied with the systems. In this paper, we emphasized the thermodynamic characteristics of the interactions between L-TyrNH_2 and its reported aptamer.[10] We varied the temperature, pH, salt concentration, and metal ion to elucidate the influence of the binding parameters on the binding behavior, especially on the binding thermodynamics. On the basis of the thermodynamics information obtained, we could possibly derive the binding mechanism.

The thermal stability of the DNA aptamer structure is dependent on salt concentration but is independent of DNA strand concentration.[18] Generally, salt concentration will influence electrostatic interactions. The literature has reported on ion effects on ligand−nucleic acid interactions. Also, utilizing the relationship between equilibrium constant (log K) and metal ion concentration (M^+) has yielded the number of charge interaction or ion pairs formed between a ligand and a nucleic acid.[19] When aptamer binds with its ligand and forms a DNA−ligand complex, the complex usually has better thermal stability than that of the original DNA.[18,20] In most cases, as an aptamer binds to its ligand, such binding is accompanied to some extent by conformational changes. Molecular dynamic simulation has demonstrated that the changes in solvent-accessible

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surface area upon complex formation have been used to estimate heat capacity changes.[18] The calculated heat capacity value agreed with the enthalpy variation studied by ITC.

The High Performance Liquid Chromatography (HPLC) system has been demonstrated to separate enantiomers (D-adenosine and L-TyrNH$_2$) by using immobilized DNA aptamers.[21] Control of the elution buffer's pH value makes the N-terminal amino group of the ligand adopt a different protonation degree in the HPLC system. However, no significant variation of retention was observed in the pH range from 5.0 to 8.0 in the HPLC system.[22] In the case of aminoglycoside−rRNA recognition processes, the ITC study demonstrated that the protonation state is a critical factor in determining the affinity of the RNA aptamer.[23] Previous studies proved that the protonation state of the drug is a critical factor in determining the affinity for its RNA aptamer.[23,24] The mechanisms of many biological systems usually involve the protonation process.

Therefore, the protonation of the ligand may play an important role in binding behavior. For this reason, some studies on protonation in binding DNA[25] and RNA[24] have been reported, and in these reports were found to correlate with the theory formula.

For other binding parameters, the influence of temperature on solute binding and chiral discrimination was adopted in the separation. To explain in greater detail, it was observed that increasing the column temperature by only ~10 °C brought about a significant reduction in the elution volume. A large exothermic enthalpy was obtained for the target enantiomer by a linear van't Hoff plot. Such a phenomenon is consistent with the indication of forming a tight complex between these analytes and the aptamers.[21] Andre et al.[26] used capillary electrophoresis and van't Hoff plots to study the binding mechanism between DNA aptamer and ligands, and adenosine and adenylate compounds. The results showed that the dehydration, van der Waals interactions, hydrogen binding, and adjustment of the aptamer recognition surface on the intermolecular binding interface were all included in the interaction.

The effects of metal ion on the structural stability of aptamer complex have been reported in the literature.[27] It deals with the effects of different cations' presence on the formation of intramolecular G-quadraplex between thrombin and its DNA aptamer. The results showed that K^+, Rb^+, NH_4^+, Sr^{2+}, and Ba^{2+} can form stable cation−aptamer complexes above 25 °C, while cations Li^+, Na^+, Cs^+, Mg^{2+}, and Ca^{2+} form weaker complexes at very low temperatures. A comparison of the thermodynamic melting profiles of aptamer's complexes with Sr^{2+} and K^+ shows that the Sr^{2+} aptamer complex is more stable. The result was mainly due to the hydration effects from the presence of cation.[27] Previous studies on the comparison of the stability effect of the aptamer complex with Na^+, Ni^{2+}, and Mg^{2+} demonstrated that the addition of Na^+ or Ni^{2+} in the buffer solution decreased the complex formation, but the addition of Mg^{2+} improved it.[26]

Recently, NMR and X-ray crystallography were used to study structural information on aptamer/ligand interactions. Correlations of the existing structural information with the binding affinity and the binding specificity to form an aptamer complex need more thermodynamic information to lend support to them. In other words, further information on structure and thermodynamics is needed for an extensive understanding of the binding parameters (pH, temperature, salt concentration, . . ., etc.) as well as the binding affinity and specificity.

Thermodynamics information on binding has mostly been studied and derived from the van't Hoff equation. The assumption of linear van't Hoff plots for enthalpy versus temperature indicates that solute and solvent properties are independent of temperature, that is, ΔH and ΔS of binding are temperature invariant for the temperature range studied. The nonlinearity of the van't Hoff plot may be attributed to the conformational changes caused by temperature, the interaction forces involved, and even the number of binding sites.[28] The above causes for the nonlinearity of the van't Hoff plot can be explained by large and negative heat capacity changes for the binding.[22] As stated, the aptamer binding with its analytes is usually accompanied by conformational change.[2,9,18,20,22,29]

The results of the van't Hoff plot by varying binding temperature for the binding enthalpy measurement may not be representative of true thermodynamics information. In this study, we performed the binding enthalpy measurement isothermally by isothermal titration calorimetry to prevent temperature effects on the binding behavior.

Isothermal titration calorimetry has been used extensively in biomolecular interactions for various applications.[18,23,30,31,69] The system studied here is L-TyrNH$_2$ with its DNA aptamer reported previously.[10] Tyrosine residue is an active site of DNA−topoisomerases that has a common mechanism of action and is characterized by nucleophilic attack onto the DNA phosphodiester backbone. These kinds of enzymes relieve the torsional stress created in DNA by processes like replication, transcription, and cell division. Vianini et al.[10] attempted to use this aptamer to elucidate the enzyme's mechanism of action and to provide some information for the discovery of novel topoisomerase inhibitors. To elucidate the mechanism of tyrosine−DNA interaction, the resolution of the tridimensional aptamer configuration is necessary. In comparison, the structure of the aptamer is still unknown, let alone the interaction mechanism. In this work, we used ITC and studied the effects of binding parameters (pH, temperature, salt concentration, and metal ion) on the binding enthalpy. The mechanism of L-TyrNH$_2$ and its DNA aptamer is discussed in detail. In addition, the protonation theory formula[25] was adopted for use in the discussion along with the ITC data obtained from this investigation.

2. Materials and Methods

Materials. DNA oligonucletides were purchased from MD-Bio, Inc. (www.mdbio.com.tw) and were used after electrophoresis purification. The 49-base aptamer sequence is 5′-AAT TCG CTA GCT GGA GCT TGG ATT GAT GTG GTG TGT GAG TGC GGT GCC C-3′. All thermodynamic experiments were performed in a buffer composed of 20 mM Tris-base, 25 mM KCl, 1.5 mM $MgCl_2$, pH 7.6. L-Tyrosinamide (L-TyrNH$_2$) and L-phenylalaninamide was obtained from Sigma Chemical Co.

Isothermal Titration Microcalorimetry. In the binding system, if one ligand binds to one binding site (1:1 stochiometry), then considering the ligand (L) contained in the solution and biomolecular (M) in the binding behavior, the binding affinity (K_A) values could be written as follows:

$$M + L \rightleftharpoons ML \quad K_A = [ML]/[M][L] \tag{1}$$

ITC experiments were done on a MicroCal VP-ITC (MicroCal Inc. Northampton, MA) with the sample cell (1.44 mL) containing the 2.5 μM DNA aptamer. The thermal equilibration step at 25 °C was followed by an initial 1200 s delay step and subsequently an initial 2 μL injection. Typically, 25 serial injections of 0.08 mM, 10 μL L-TyrNH$_2$ at intervals of 300 s were made with continuous stirring at 310 rpm of the solution in the sample cell. Raw data as power (μcaL/s) vs time (min)

were recorded. The heat associated with each titration peak was integrated and plotted against the respective molar ratio of L-TyrNH$_2$ and DNA aptamer. The resulting experimental binding isotherm was corrected for the effect of titrating L-TyrNH$_2$ into binding buffer. Thermodynamic parameters were extracted from a curve fit to the corrected data by using the one-site binding model (eq 2) in the software (Origin 5.0) provided by MicroCal (MicroCal LLC). The changes in enthalpy ΔH and the binding affinity K_A were obtained:

$$q_i = \upsilon \Delta H[P]\left\{\frac{K_A[L]_i}{1+K_A[L]_i} - \frac{K_A[L]_{i-1}}{1+K_A[L]_{i-1}}\right\} \quad (2)$$

where q is the measured amount of heat released, υ is the known value of the reaction, and $[L]_i$ is the concentration of ligand at the i^{th} injection.[32] Consequently, the changes in Gibbs free energy (ΔG) and the changes in entropy (ΔS) can be calculated from eqs 3 and 4, where T is the reaction temperature (in K) and R is the gas constant (1.986 cal K^{-1} mol^{-1}).

$$\Delta G = -RT\ln(K_A) \quad (3)$$

$$\Delta G = \Delta H - T\Delta S \quad (4)$$

Circular Dichroism Spectroscopy. Circular dichroism spectra were recorded on a Jasco J-810 spectropolarimeter (Jasco, Inc., Easton, MD) interfaced with a computer and equipped with a heating/cooling device and nitrogen purging facilities. The CD spectrum of the 2.5 μM DNA aptamer was recorded from 310 to 220 nm; the data gathered were the average of four time scans at a scanning rate of 100 nm/min. The scan of the buffer alone recorded at room temperature was substracted from the average scans for each DNA duplex. The data were collected in units of millidegrees versus wavelength and were normalized to total DNA concentration.

3. Results and Discussion

ITC provides useful thermodynamic information in studying the binding processes involving proteins and ligands in a solution. It allows direct measurement of enthalpy changes (ΔH) associated with biochemical reactions and processes isothermally.

To say that the ΔH for binding interaction approaches zero as the system nears equilibrium requires a sensitive probe on the extent of the binding interaction. Subtraction of the heat of dilution for a given binding reveals the binding enthalpy, and along with the provided binding isotherm, the binding affinity (K_A), molar based binding enthalpy, and binding entropy can be calculated.

The molecular structure of the ligand (L-TyrNH$_2$) includes one amino, one amide, one phenyl, and one phenolic hydroxyl group. Therefore, it is possible that there are many interaction forces involved in this aptamer binding mechanism. In this study, we discussed the binding behavior at different temperatures, salt concentrations, pH values, analogues, and magnesium cations. Along with the coupled CD and ITC studies, it is expected that we can gain a better understanding on the recognized mechanism and structural information of aptamer and ligand.

Circular Dichroism Studies. To investigate the secondary structural change of the DNA aptamer upon binding, the CD spectra of the DNA aptamer were titrated with various concentrations of L-TyrNH$_2$. The CD spectra are shown in Figure 1. The CD spectrum of the pure DNA aptamer displays a negative band at 245 nm and a positive band at 280 nm, which are the characteristics of B-DNA form.[34] The CD spectrum of the L-TyrNH$_2$ and aptamer complex was blue-shifted and had an increased positive band near 265 nm and a negative band near 240 nm. A typical A-form DNA displays a negative CD peak at 240 nm followed by a positive peak at 265 nm, while B-form CD bands are much weaker than A-form CD bands. The CD spectra indicate that the binding of L-TyrNH$_2$ to DNA aptamer induces a secondary structure change from B-form to A-form. The effect of temperature on CD was studied in this investigation. The results in CD spectra are discussed with the ITC data as follows. Interestingly, when the L-TyrNH$_2$ and aptamer concentration ratio is 1:1, the spectrum hardly changes with the initial aptamer DNA at 30 °C. By increasing the concentration of L-TyrNH2 to 5:1, the DNA structure changed. Perhaps the increase in concentration ratio resulted in an increased probability of the molecules contacted at the same time. However, when the temperature rises to 40 °C, the CD spectrum has not been changed with increasing the concentration of L-TyrNH2 as shown in Figure 2A−C. When the increase in temperature is from 15 to 40 °C, there is no change in the CD spectra of the DNA aptamer, whereas L-TyrNH$_2$ indicates a zero value showing that the DNA structure changes depending on the binding of L-TyrNH$_2$ (Figure 2D). To discuss the aptamer binding mechanism thoroughly, it is necessary to integrate it with more thermodynamics information from ITC.

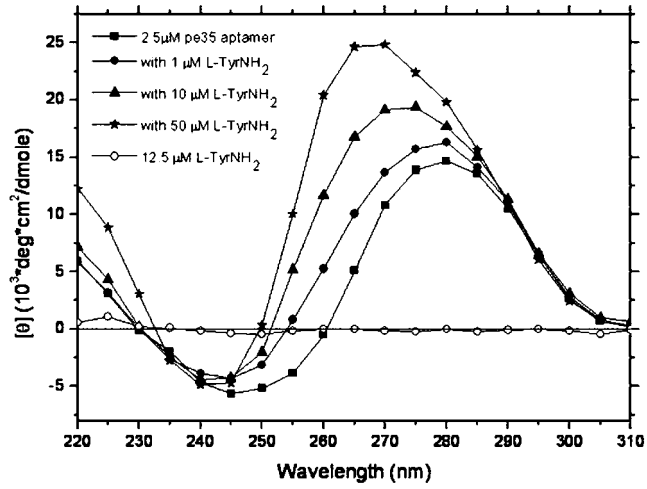

Figure 1. The CD spectra of the 2.5 μM pe35 DNA aptamer was titrated with various concentrations of L-TyrNH$_2$ at 20 mM Tris base, 25 mM KCl, 1.5 mM MgCl$_2$, pH 7.6, 25 °C.

The Temperature Effect. To confirm whether the hydrophobic effect in this binding mechanism is possible, we utilized 2.5 μM DNA aptamer and titrated it with 0.08 mM L-TyrNH$_2$ in the ITC system. The derived disassociation constant (K_d) is 16.8 μM at 25 °C (Figure 3 and Table 1). Vianini et al.[10] utilized affinity chromatography and equilibrium dialysis to estimate the K_d and obtained $K_d = 45$ μM. Merino et al.[35] utilized modulation of the local environment at an appropriate fluorescent group, tethered via a 2′-amide linkage, and obtained the $K_d = 20$ μM. Our result of our study has the same order with previous investigations which indicate that the binding affinity calculated by ITC is reasonable. Therefore, this binding mechanism reveals a highly favorable enthalpy of reaction (ΔH) at 25 °C of –20.1 kcal mol^{-1} offset by a large and unfavorable entropy of reaction ($T\Delta S$ =−12.6 kcal mol^{-1}) as shown in Table 2. These thermodynamic and conformational data, particularly the large negative $T\Delta S$ and conformation change, likely reflect the local folding of events in the aptamer that occur during ligand binding; this is a common feature in RNA−protein interactions.[36,37] In recent years, many reports found that there has been an induced-

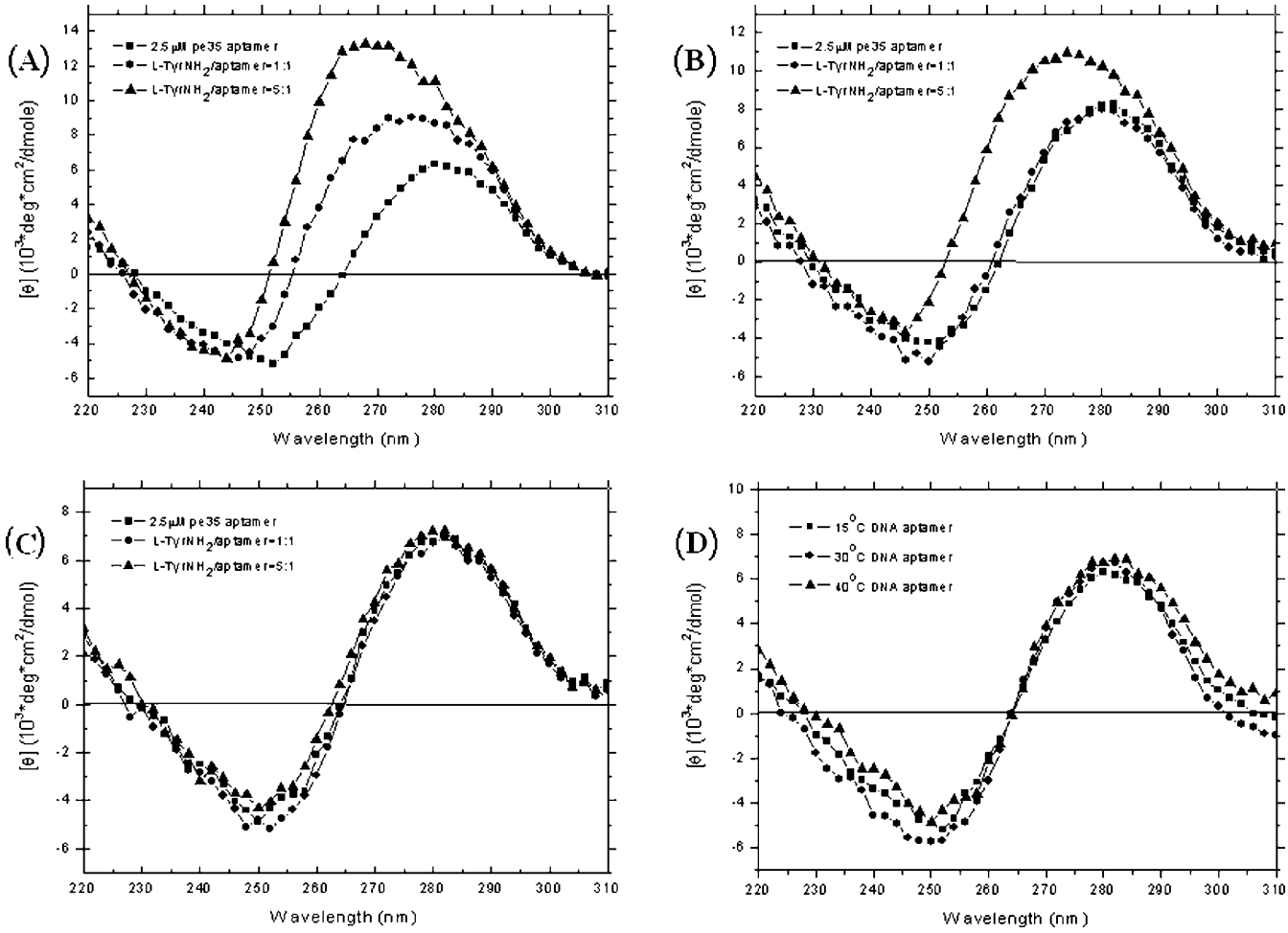

Figure 2. The CD spectra of the 2.5 μM pe35 DNA aptamer with different L-TyrNH$_2$ concentration ratios at 20 mM Tris base, 25 mM KCl, 1.5 mM MgCl$_2$, pH 7.6: (A) 15, (B) 30, and (C) 40 °C; (D) aptamer at different temperatures in the absence of L-TyrNH$_2$.

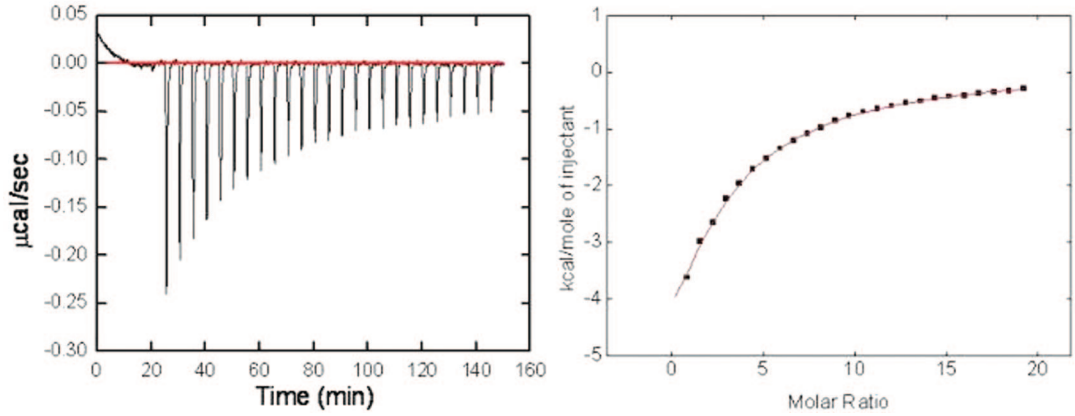

Figure 3. The ITC profiles at 20 mM Tris base, 150 mM NaCl, 1.5 mM MgCl$_2$, pH 7.6, 25 °C.

TABLE 1: The Thermodynamic Parameter at 20 mM Tris Base, 150 mM NaCl, 1.5 mM MgCl$_2$, pH 7.6, 25 °C from the ITC Result

K_A (1/M)	K_d (mM)	ΔH (kcal/mol)	ΔG (kcal/mol)	ΔS (cal/(mol·K))	$-T\Delta S$ (kcal/mol)
$(6.0 \pm 0.06) \times 10^4$	16.8	-31.1 ± 0.2	-6.51	-82.4	24.5

fit process in the small molecule−aptamer binding mechanism.[30] The aptamer binding pocket is more random (disorder) before binding with L-TyrNH$_2$, but after the formation of the aptamer/L-TyrNH$_2$ complex, the binding pocket changed to a well-defined state that is probably responsible for unfavorable entropy.[18,30,38] Further, the overall entropy change in the binding system is contributed from the DNA conformation, and from the various dispositions of solvent–water, and from the molecule translational and conformational freedom and counterion molecules.[33,39,40] The contribution to the observed entropy

TABLE 2: The Thermodynamic Parameter at 20 mM Tris Base, 25 mM KCl, 1.5 mM $MgCl_2$, pH 7.6, 15−30 °C from the ITC Result

	15 °C	20 °C	25 °C	30 °C
K_A (1/M)	$(9.4 \pm 0.2) \times 10^5$	$(5.7 \pm 0.1) \times 10^5$	$(3.1 \pm 0.06) \times 10^5$	$(4.9 \pm 0.05) \times 10^4$
ΔH (kcal/mol)	-15.2 ± 0.09	-17.9 ± 0.1	-20.1 ± 0.2	-98 ± 0.7
ΔS (cal/(mol·K))	−25.3	−34.6	−42.3	−304.2
ΔC_p (cal/mol)		−495		

change from the counterion effect was estimated to be weak.[40] Previous studies have shown that the reduction of translational and rotational degrees of freedom can also be neglected.[41] Accordingly, the major contribution to the entropy change would result mainly from the conformational and dehydration entropy change. From this reason, we can derive that the conformational change plays an important role in this binding system. Induced-fit is classically associated with complex formation in which both components are able to adjust their recognition surfaces to maximize complementary through tightly packed contacts involving stacking, electrostatic interactions, and hydrogen bonding.[22] Many riboswitches control gene expression through their aptamer domain conformation change,[2] and many aptamers' interaction with their ligand also involves this phenomenon.[18,21,42] To explore whether aptamer-L-TyrNH$_2$ binding displays all the thermodynamic hallmarks consistent with an induced-fit binding mechanism, we determined the heat capacity change (ΔC_p) of reaction. Heat capacity change is generally related to hydration change. Dehydration of the polar surfaces is a process accompanied by a positive heat capacity change. Relatively, a major negative heat capacity change was observed for the dehydration of nonpolar surfaces.[33,40,43] In an induced-fit process, the binding mechanism makes the local folding behavior decrease the nonpolar solvent accessible area of the biomacromolecule (such as aptamer and protein) and ligands, which causes the heat capacity to decrease after the binding process. For this reason, the heat capacity change (ΔC_p) had a large negative value.[30,37] It should be noted that the binding affinity (K_A) was one magnitude order lower at 30 °C as compared to that at other temperatures (Figure 4A). This phenomenon at 30 °C is similar with the CD spectra result. It was inferred from this phenomenon that the binding system perhaps had changed at 30 °C. At a high temperature, the DNA aptamer had higher kinetic energy, which causes higher initial entropy of the system. Therefore, at 30 °C when the L-TyrNH$_2$ and aptamer concentration ratio is 1:1, overcoming the unfavorable entropy that causes the different phenomenon is difficult. The L-TyrNH$_2$ binding to the aptamer needs to overcome a large amount of decrease in the entropy of the system. Therefore, this could explain the affinity and entropy decrease at a high temperature.

In this study, we utilized the binding enthalpies at different temperatures (15−25 °C) to calculate the heat capacity change. A large and negative heat capacity change ΔC_p ($\Delta C_p = -0.495$ kcal/(mol·K)) was observed at this binding system (Figure 4 B). Coupling the CD spectra with ITC, the conformational changes influenced the ΔC_p and ΔS, which could be regulated from the folding appearance interpreted by the excess in ΔC_p and the deficit in ΔS.[44] The heat capacity change suggests that there is a plausible conformational structural transition that occurs in the aptamer sequence upon complex formation.[18] According to the results of our study and literature data, we strongly predict that there is an induce-fit process in this binding system. Aside from the large and negative heat capacity change, ΔC_p implies that there was a hydrophobic effect involved in this binding mechanism. From the functional groups of the L-TyrNH$_2$, the phenyl group could contribute to the hydrophobic effect in base stacking. This inference is identical with that of Hermann et al. that the precise stacking of flat moieties is a very important driving force in aptamers' interaction with small molecules.[42]

The Salt Concentration Effect. To confirm whether electrostatic interaction is involved or not, the effect of salt concentration of the system was studied. The nucleic acid and ligand interaction are often included with electrostatic interaction and protonation processes.[24,25] The electrostatic interaction between a small molecule and the nucleic acid target is the driving force for binding and has been explored as a means of affinity enhancement.[22,23,25,45,46] From the results of ITC, K_A was found to decrease with an increasing Na^+ concentration (Table 3). This observation indicates that this aptamer binding mechanism includes electrostatic interactions. Combined with the CD spectra, we could observe that there is a small peak shift with increased Na^+ concentration (Figure 5B). In the ITC results, the binding affinity is decreased with an increase in the

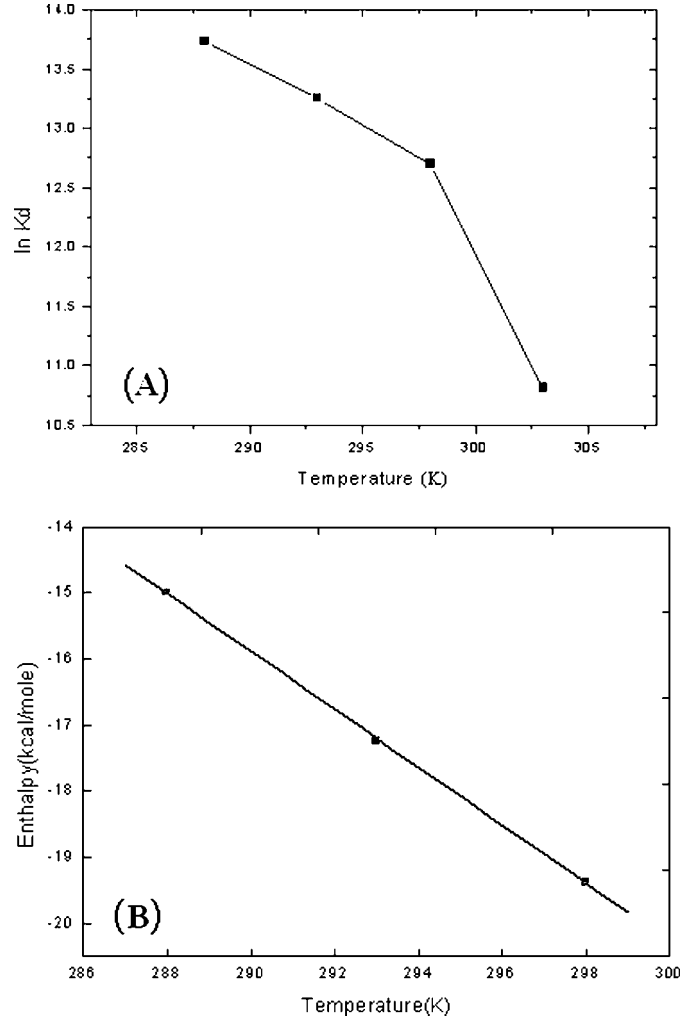

Figure 4. (A) Plot of ln K_d vs temperature and (B) plot of ΔH vs temperature.

TABLE 3: The Thermodynamic Parameter at 20 mM Tris Base, 1.5 mM $MgCl_2$, pH 7.6, 25 °C, 25−150 mM NaCl from the ITC Result

	25 mM Na^+	50 mM Na^+	100 mM Na^+	150 mM Na^+
K_A (1/M)	$(4.1 \pm 0.09) \times 10^5$	$(1. \pm 0.03) \times 10^5$	$(8.8 \pm 0.07) \times 10^4$	$(6.0 \pm 0.06) \times 10^4$
ΔH (kcal/mol)	-23.7 ± 0.2	-21.4 ± 0.2	-29.0 ± 0.1	-31.1 ± 0.2
ΔS (cal/(mol·K))	−53.9	−47.7	−76.7	−82.4

salt concentration. This indicates that at higher salt concentration the binding Takes place with more difficulty, which causes a decrease in the binding affinity. The CD spectra show that at a higher salt concentration, the binding behavior decreases, which causes the reduced peak shift as compared to a lower salt concentration. Similar spectral behavior was also observed in KCl solution.

In the literature, the following equation was proposed to measure the contribution of electrostatic interaction in the binding mechanism:[19]

$$\frac{\partial \log K_A}{\partial \log [Na^+]} = -Z\varphi \tag{5}$$

In this equation, Z is the apparent charge on the bound ligand, and φ is the fraction of Na^+ bound per nucleic acid phosphate. The value of φ for the single-stranded DNA is 0.71, while that for native DNA is 0.88.[19] Aptamer usually folds into a secondary structure having the pair and nonpair regions in that the φ value

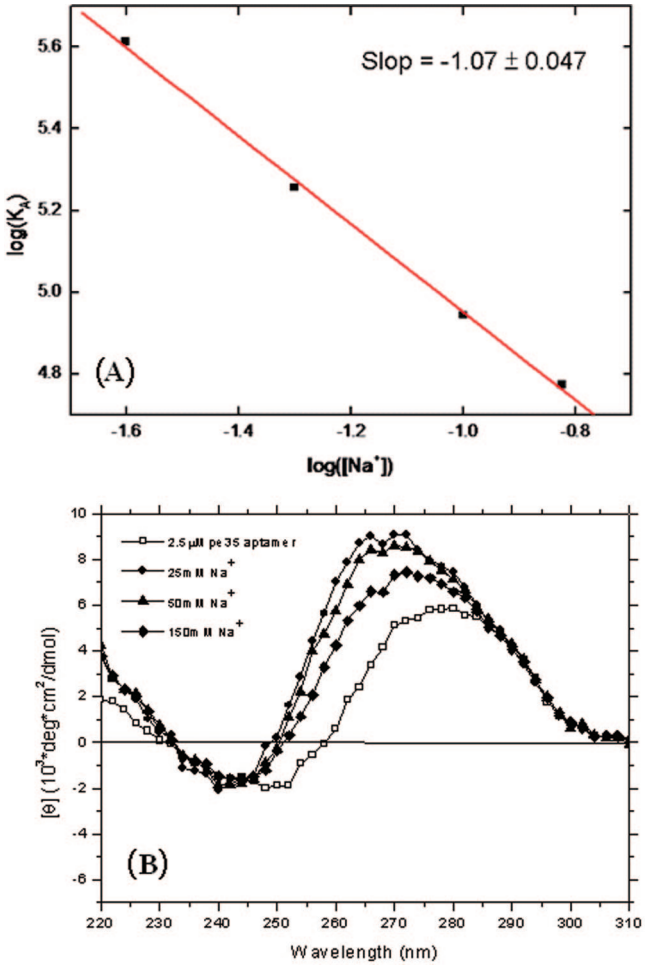

Figure 5. (A) Plots of log K_A vs log Na^+ and (B) CD spectra of the DNA aptamer and L-$TyrNH_2$ concentration ratio (5:1) at 20 mM Tris base, 1.5 mM $MgCl_2$, 25 °C, pH 7.6, and different salt (Na^+) concentrations.

should be intermediate between these two values.[23,46] From plots of log K_A vs log [Na^+], we can estimate the minimum number of NH_3^+ groups that participate in the electrostatic interactions with the nucleic acid. The slope is –1.07 ± 0.047 (Figure 5A), indicating that at least one NH_3^+ group is involved in the electrostatic interaction.

The portion of free energy associated with electrostatic interactions can be determined by the following equation:[19]

$$\Delta G_{elec} = Z\varphi RT \ln [Na^+] \tag{6}$$

where $Z\varphi$ is the slope determined through linear regression analysis, and ΔG_{elec} is the electrostatic interactions' contribution to the observed binding free energy.

Comparing the total free energy of binding with the electrostatic contribution, it appears to be about one-quarter of the total free energy of binding from the electrostatic interactions in the presence of various concentrations of Na^+. This result implies that there would be another interaction force included in this binding mechanism, such as hydrogen bond, van der Waals force, and hydrophobic effect.

On the aspect of thermodynamics, the binding enthalpy and entropy trend decreases with an increase in salt concentration. However, Cowan et al.[47] used ITC to investigate the thermodynamics of neomycin B and cognate RNA aptamer binding behavior. They obtained different results. They found that the binding enthalpy and entropy are increased with salt concentration. In previous studies, it was reported that there are two kinds of forces which stabilize the structure of DNA, one is the hydrophobic effect in base stacking and the other is the electrostatic repulsion of the phosphate backbone.[48–50] DNA flexibility is increased as the salt concentration is increased, owing much to the charge neutralization between phosphate backbones.[50,51] In neomycin B and cognate RNA aptamer binding behavior, the structure of RNA does not change much after ligand binding. In this study, however, we observed that the conformational change is a very important phenomenon in this binding mechanism from the CD spectra. The increase in concentration influences the electrostatic interaction mainly in neomycin B and the cognate RNA aptamer binding mechanism. However, in this study, aside from the influence of electrostatic interaction, a reduced influence of DNA flexibility was observed because of electrostatic repulsion. The system entropy at a high salt concentration is larger than that at a lower salt concentration before aptamer binding with L-$TyrNH_2$. After binding with L-$TyrNH_2$, the system entropy was reduced, and the complex exhibited the same entropy value at different salt concentrations. Combining the system entropy overall, the system entropy change is negative, and the entropy change at a high salt concentration is less than that at a low salt concentration. Furthermore, at a high salt concentration, the DNA flexibility increases, which results in a decrease in the energy of conformational change. The enthalpy change of the binding system at a high salt concentration is smaller than that at a low salt concentration.

Generally, the basic structure of the DNA is maintained by base stacking due to hydrophobic effects[52] and electrostatic

TABLE 4: The Thermodynamic Parameter at 20 mM Tris Base, 25 mM KCl, 1.5 mM $MgCl_2$, 25 °C, pH 5−9 from the ITC Result

	pH 5	pH 6	pH 7.6	pH 9
K_A (1/M)	$(6.2 \pm 0.1) \times 10^5$	$(6.6 \pm 0.1) \times 10^5$	$(3.2 \pm 0.4) \times 10^5$	nf
ΔH (kcal/mol)	-8.3 ± 0.1	-17 ± 0.1	-20.1 ± 0.2	nf
ΔS (cal/(mol·K))	−1.497	−26.29	−39.75	nf

repulsion between the phosphate groups of the backbone.[53] At a high salt concentration, the ion has covered the electrostatic effect that resulted in a comparatively loose phosphate backbone.[51] Thus, the entropy of the aptamer at a high salt concentration is higher than that at a low salt concentration before binding. After forming a complex with L-TyrNH$_2$, however, the structure will become comparatively rigid. Therefore, the entropy change at high and low salt concentrations will be similar in the binding process. At a high salt concentration, dehydration enthalpy is less required as compared to that at a low salt concentration.

The pH Effect and the Binding-Linked Protonation Process. From the salt concentration effect, it can be deduced that there was an electrostatic interaction, and therefore at least one NH_3^+ group was involved in this binding mechanism. The electrostatic interaction may include the binding-linked protonation process.[23–25,54–56] The different pH values could give protonation information and the contribution of amino and amide groups in this binding mechanism. The pK_a of the amide group is 7.7[57] and that of the amino group is 9.11. When the pH value is lower than 7.7, the amide and amino groups will be protonated, and the molecule has a positive charge. At pH 9, the amide group will be almost deprotonated, and half of the amino groups will be protonated. For this reason, whether they are a main contributor in electrostatic interaction to judge amide protonation at pH 9, the different pH values could give some protonation information and the contribution of these two groups in this binding mechanism. From ITC and CD spectra, we inferred that the binding is suppressed at pH 9 (Table 4). Furthermore, from CD spectrum, we could not find the peak shift at pH 9 (Figure 6). We recall the pK_a values of these two functional groups in L-TyrNH$_2$ as 7.7 and 9.11. Thus, increasing pH values above 7.7 will reduce the extent to which the amide group on L-TyrNH$_2$ is protonated. When the pH value is increased, the system binding affinity decreases, and the binding enthalpy becomes more exothermic (favorable), while the entropic contribution to binding becomes unfavorable. Thus, the pH-induced reduction in ΔG is entirely entropic in origin. This phenomenon agrees with the coupling of binding to the protonation effect.[32,56] In this connection, we sought to determine whether the pH dependence of the observed binding properties described above reflects a linkage between L-TyrNH$_2$ bindings and the release or uptake of protons. We conducted ITC experiments at pH 7.6 using buffers that differ with respect to their heats of ionization (ΔH_i). Thus, if the pH dependence of ΔH_{obs} reflects binding-induced uptake or release of protons, then its value at a given pH should vary with the ΔH_i value of the buffer.

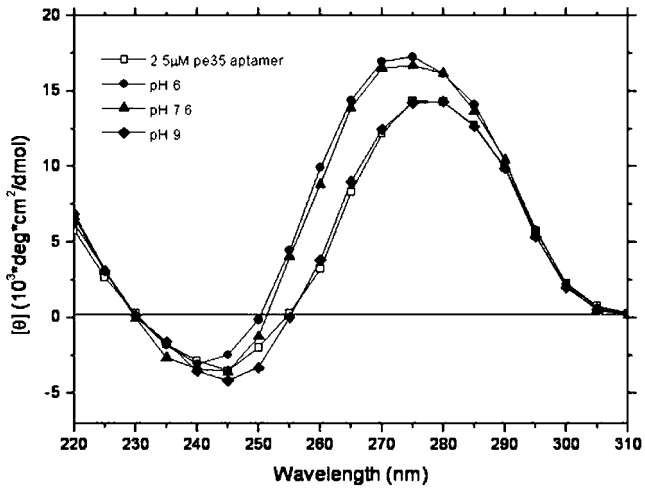

Figure 6. The CD spectra of the DNA aptamer and L-TyrNH$_2$ concentration ratio (5:1) at 20 mM Tris base, 25 mM KCl, 1.5 mM $MgCl_2$, 25 °C, and different pH values.

TABLE 5: The Enthalpy Change in Different Buffer Systems (20 mM Tris, phodphate) at 25 mM KCl, 1.5 mM $MgCl_2$, pH 6 and 7.6, 25 °C from the ITC Result

ΔH (kcal/mol)	pH 6	pH 7.6
Tris buffer	-17.0 ± 0.1	-20.1 ± 0.2
K-phosphate buffer	-16.9 ± 0.2	-24.2 ± 0.3

Furthermore, the number of protons linked to binding at a specific pH (n_{cal}) as well as the intrinsic binding enthalpy (ΔH_{int}) combine with the heat of ligand protonation (ΔH_{LP}); a value that differs from ΔH_{obs} in that it excludes enthalpic contributions from ionization of the buffer can be determined by simultaneous solution of the following equation:[25]

$$\Delta H_{obs} = \Delta H_{int} + n_{cal}(\Delta H_{LP} + \Delta H_i) \quad (7)$$

At pH 6, the two functional groups of L-TyrNH$_2$ are protonated and will not have the binding-linked protonation process. We then probed for coupled protonation or deprotonation reactions to this binding system using ITC in two different buffers (Tris and phosphate) that exhibit differing heats of dissociation of the protonation (45.6[58] and 5.12[59] kJ/mol, respectively). Note that the values of ΔH_{obs} are essentially identical in these two buffers (Table 5). This result indicates that there is an absence of protonation effects at pH 6 in this binding behavior.

For a system of two buffers at the same pH, the number of moles of protons linked per mole of complex formation can be evaluated as

$$n_{cal} = (\Delta H_{obs}(1) - \Delta H_{obs}(2))/(\Delta H_i(1) - \Delta H_i(2)) \quad (8)$$

Thus, at an increase in pH value to 7.6, the protonation of the system is 0.42. This indicates that there is almost 50% of protonate involved in this process. This is because the pH value is the pK_a value of the amide group among the L-TyrNH$_2$s that have a 50% amide group that has not yet been protonated. From this result, we strongly predict that the amide group protonation for this binding behavior is essential. The binding-linked protonation process involved in the interaction of aptamers and ligands not only enhances the stability of thermodynamics but also welcomes the possibility of hydrogen bond formation. This can perhaps improve the specificity of aptamers.

The Different Analogues. Furthermore, L-TyrNH$_2$ and an analogue were also compared to understand the contribution of the phenolic hydroxyl group. To better understand the mechanism of aptamer binding to L-TyrNH$_2$ and what the functional

TABLE 6: The Thermodynamic Parameter of the Interaction of Different Ligands (L-TyrNH$_2$, L-PheNH$_2$) with Aptamer at 20 mM Tris Base, 25 mM KCl, 1.5 mM MgCl$_2$, pH 7.6, 25 °C from the ITC Result

	L-TyrNH$_2$	L-PheNH$_2$
K_A (1/M)	$(3.1 \pm 0.06) \times 10^5$	$(4.0 \pm 0.1) \times 10^4$
ΔH (kcal/mol)	-20.1 ± 0.2	-9.1 ± 0.2
ΔS (cal/(mol·K))	-42.3	-9.56

group of L-TyrNH$_2$ is, we also checked the aptamer selectivity for L-TyrNH$_2$ by ITC with L-phenylalanamide (L-PheNH$_2$). Initially, we compared the affinity with L-TyrNH$_2$ and L-PheNH$_2$. The affinity of L-TyrNH$_2$ is much higher than that of L-PheNH$_2$ at approximately one magnitude of order (Table 6). From the CD spectrum data, we have observed a smaller CD peak shift for L-PheNH$_2$ as compared to that of L- TyrNH$_2$ (Figure 7). Only one phenolic hydroxyl group is different from these two ligands. Hence, this hydroxyl group appears to direct specificity to a large extent. From the enthalpy, L-TyrNH$_2$ exhibited more heat release. In other words, L-TyrNH$_2$ had more favorable enthalpy to interact with aptamer. Therefore, it was inferred that L-TyrNH$_2$ perhaps has a greater interaction force than L-PheNH$_2$ to bind aptamer, and this results in higher affinity and exothermic behavior. For the entropy, L-TyrNH$_2$ had a greater interaction force, which resulted in unfavorable entropy. It is conjectured that the extra interaction force is hydrogen bonding given the description of the above data. However, Vianini et al.[10] used affinity chromatography and equilibrium dialysis to analyze the same aptamer and ligands (L-TyrNH$_2$ and L-PheNH$_2$) and obtained a different result. They found that the K_d value for L-PheNH$_2$ is only twice the value for L-TyrNH$_2$. Assuming that phenolic hydroxyl does not have a direct effect on specificity, there is a need to rule out the hydrogen bonding contribution to complex stability. This is unexpected, and the results are contradictory to the present work. The different binding systems will affect the affinity of the aptamer; this means that the selected aptamers favor the free ligand conformation over the matrix-bound conformation.[60]

The Mg^{2+} Effect. DNA−metal ion interactions play a key role in the control of DNA conformation and its topology.[61] Different studies have demonstrated that ligand−aptamer complex formations are governed by slow mass transfer kinetics and that magnesium cation stabilizes the aptamer secondary structure.[62] Not only can the magnesium cation stabilize the aptamer secondary structure[30,38,63,64] but it can also help in conformational changes[38,65,66] and the ligand−aptamer complex formation.[63] In this study, thermodynamics (ITC) and conformational information (CD) were integrated to investigate the impact of magnesium on the binding mechanism. These experiments were carried out for different magnesium concentrations in the system (Table 7). In the absence of magnesium cation, there was no exothermic behavior from ITC, and any shift in the CD spectrum could not be found. The binding affinity increased with the concentration of Mg^{2+} ions. This result is in agreement with previous reports in which Mg^{2+} was indispensable in the formation of the ligand−aptamer complex and could enhance the binding affinity.[26,30,38,63,64,67,68] Interestingly, it is observed that the CD spectra of pure DNA aptamer were shifted with Mg^{2+}, indicating that the Mg^{2+} will induce the conformational change of the DNA aptamer (Figure 8). From the ITC results, when the concentration of Mg^{2+} is increased, the binding ΔH and ΔS tend to be more positive, implying that the thermodynamic drive of the binding system is shaded. From the experimental results of ITC and CD, we inferred boldly that Mg^{2+} is very much needed to stabilize the DNA aptamer. This also brings about the conformational changes of the DNA aptamer. However, it is necessary to provide more heat to adjust the conformation in binding behavior, which reduces the unfavorable entropy to drive the binding mechanism.

TABLE 7: The Thermodynamic Parameter at 20 mM Tris Base, 25 mM KCl, pH 7.6, 25 °C, 1.5 and 5 mM MgCl$_2$ from the ITC Result

	1.5 mM	5 mM
K_A (1/M)	$(3.1 \pm 0.06) \times 10^5$	$(5.5 \pm 0.1) \times 10^5$
ΔH (kcal/mol)	-20.1 ± 0.2	-9.2 ± 0.07
ΔS (cal/(mol·K))	-42.3	-4.69

4. Conclusion

Summarizing the above, we presume that the binding mechanism of aptamer and L-TyrNH$_2$ is an enthalpy-driven process. The major contribution to the entropy change would result mainly from the conformational and dehydration changes, and a high negative heat capacity change was contributed by the dehydration of nonpolar surfaces. From the large, negative value of entropy, heat capacity change, and CD spectra, we could conclude that the binding mechanism between the aptamer and

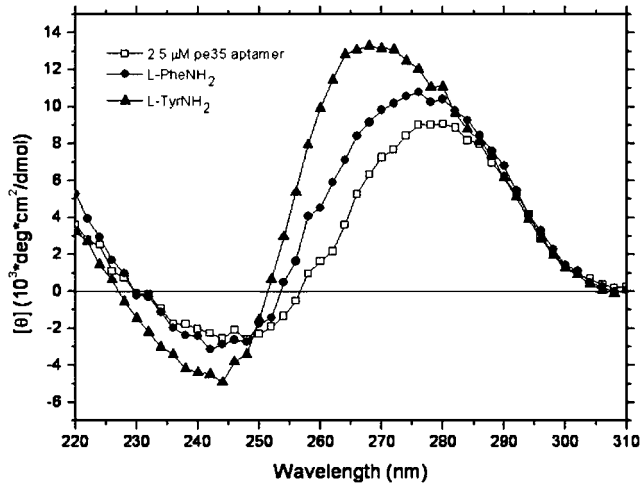

Figure 7. The CD spectra of the interaction with different ligands (L-TyrNH$_2$, L-PheNH$_2$) with aptamer at 20 mM Tris base, 25 mM KCl, 1.5 mM MgCl$_2$, pH 7.6, 25 °C.

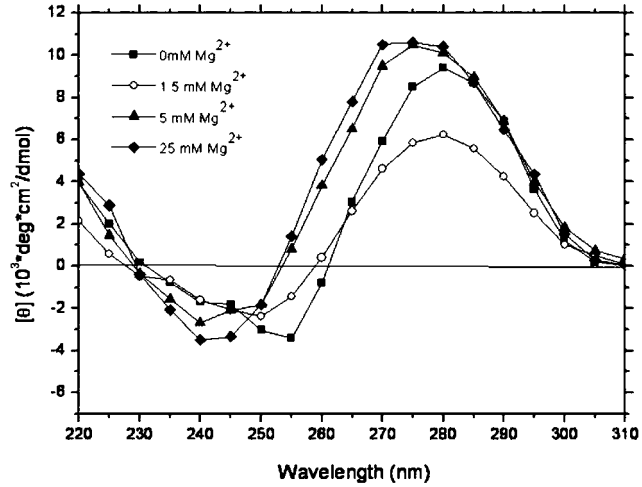

Figure 8. The CD spectra of the DNA aptamer at 20 mM Tris base, 25 mM KCl, 25 °C, pH 7.6, and different Mg^{2+} concentrations.

the ligand is an induced-fit process. Specifically, the phenyl group of L-TyrNH$_2$ contributes to the hydrophobic effect in the base stacking. The NH_3^+ groups are involved in the electrostatic interactions, and the binding-linked protonation process of the amide group is essential for binding behavior. Furthermore, the phenolic hydroxyl group of the L-TyrNH$_2$ may offer a hydrogen bonding to stabilize this aptamer/ligand complex.

This identified binding behavior induces aptamer dehydration, which resulted in the structural changes from B-form to A-form. This observed special phenomenon has not yet been reported previously. The ITC and CD results revealed that the magnesium ion not only contributes to the binding interaction on aptamer and L-TyrNH$_2$ but also influences the conformation of the aptamer.

This study proposes novel approaches to the interaction mechanism study between aptamer and ligand. The results also provide supportive information for structural investigation from NMR and X-ray crystallography.

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