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Studies of the binding mechanism between aptamers and thrombin by circular dichroism, surface plasmon resonance and isothermal titration calorimetry

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ABSTRACT

Thrombin, a multifunctional serine protease, has both procoagulant and anticoagulant functions in human blood. Thrombin has two electropositive exosites. One is the fibrinogen-binding site and the other is the heparin-binding site. Over the past decade, two thrombin-binding aptamers (15-mer and 29-mer) were reported by SELEX technique. Recently, many studies examined the interactions between the 15-mer aptamer and thrombin extensively, but the data on the difference of these two aptamers binding to thrombin are still lacking and worth investigating for fundamental understanding. In the present study, we combined conformational data from circular dichroism (CD), kinetics and thermodynamics information from surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC) to compare the binding mechanism between the two aptamers with thrombin. Special attentions were paid to the formation of G-quadruplex and the effects of ions on the aptamer conformation on the binding and the kinetics discrimination between specific and nonspecific interactions of the binding. The results indicated reasonably that the 15-mer aptamer bound to fibrinogen-binding site of thrombin using a G-quadruplex structure and was dominated by electrostatic interactions, while the 29-mer aptamer bound to heparin-binding site thrombin using a duplex structure and was driven mainly by hydrophobic effects.

1. Introduction

In 1990, an important technique emerged, using in vitro selection approaches to isolate RNA and DNA molecules that bind nucleic acids binding proteins [1–3]. The in vitro selection procedure was called "systemic evolution of ligands by exponential enrichment" (SELEX), and the products were named "aptamers". Single stranded DNA (ssDNA) and RNA aptamers selectively bind to their target molecules and offer molecular recognition properties rivaling that of antibodies, and thus are useful in biotechnological and therapeutic applications.

Any vascular injury causes the release of the trypsin-like serine protease α-thrombin. Thrombin is composed of a serine protease domain and can diffuse to encounter many protein substrates. The concentration of thrombin in blood indicates the physiological function of human body. The crystal structure of thrombin solution revealed an active site and two exosites, and utilize these features to recognize substrates [4]. In most studies, both thrombin exosites either directly interact with the substrate protein or indirectly interact with a third cofactor molecule [5]. Exosite I is the fibrinogen recognition exosite. The center of this exosite is a hydrophobic region. Exosite II is the putative heparin-binding site of thrombin and has a hydrophobic notch. There are many positive charge residues around these two exosites. The basic information emerging from recent structural, mutagenesis and kinetic investigation of thrombin shown that thrombin exists in three forms, inactive form, original enzyme form and enzyme coupling with Na^+ form, that interconvert under the influence of ligand binding to distinct domains. The complexity of function and regulation has caused thrombin as the prototypic allosteric serine protease. Thrombin is now looked upon as a model system for the quantitative analysis of biologically important enzymes [6].

In 1992, Bock et al. [7] used SELEX to develop a 15-mer ssDNA aptamer for thrombin. The use of the 15-mer aptamer to regulate the activity of thrombin or to detect the concentration of thrombin has been an important topic in recent years. Most studies on the 15-mer aptamer focused on how to optimize the detection platform. In 2001, Hamaguchi et al. [8] turned a 15-mer aptamer into a 20-mer aptamer beacon by adding nucleotides to the 5′-end complementary to the nucleotides at the 3′-end of the aptamer. Similar

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Table 1
Aptamer sequence.

Abbreviation	Sequence
15-mer aptamer	5′-GGT TGG TGT GGT TGG-3′
20-mer aptamer	5′-GGT TGG TGT GGT TGG CAA CC-3′
29-mer aptamer	5′-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3′
32-mer aptamer	5′-GG TAG GGC AGG TTG GGT GTT TTC ACT TTT GGG-3′

to molecular beacons can adopt two or more conformations, one of which allows ligand binding. A fluorescence-quenching pair is used to detect changes in conformation induced by ligand binding. 20-mer aptamer beacons provided a new class of molecules for detecting a wide range of ligands.

However, Tasset et al. [9] used SELEX and developed a 29-mer aptamer for thrombin. Ostatna et al. [10] modified the 29-mer aptamer to a 32-mer aptamer by adding a random sequence on 3′-end to avoid self-hybridization of DNA. Immobilized on a SPR sensor to detect thrombin, the 32-mer aptamer yields best results both in sensor specificity and in sensitivity.

Despite of many studies reported how to optimize the applications of the 15-mer aptamer [11], the binding mechanism of the 15-mer aptamer with its target ligand has not been elucidating in depth. Moreover, the binding mechanism of 29-mer aptamer is also needed to be clear and compared with that of 15-mer aptamer.

Therefore, the present study, we used CD to study the effects of binding parameters (salt concentration, metal ion, thrombin concentration) on the aptamers' conformation change. The kinetic parameters were analyzed by SPR and the thermodynamics results from ITC, the mechanism of thrombin and its aptamers (15-mer and 29-mer) were discussed. These results were then used to infer the mechanism of thrombin interacting with the two aptamers.

2. Materials and methods

2.1. Materials

DNA oligonucleotides were purchased from MDBio, Inc. (www.mdbio.com.tw) and were purified by electrophoresis. Table 1 illustrates the aptamer sequences. The 15-mer aptamer reaction was performed in 15-mer SELEX buffer (Buffer I: 20 mM Tris–HCl, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4), while the 29-mer aptamer reaction was performed in 29-mer SELEX buffer (Buffer II: 20 mM Tris–HCl, 100 mM NaCl, 1 mM $MgCl_2$, pH 7.4) and Tris–HCl buffer pH 7.4. Thrombin was obtained from Sigma (USA).

2.2. Circular dichroism spectroscopy

Circular dichroism spectra were recorded on a Jasco J-810 spectropolarimeter (Jasco, Inc., Easton, MD) interfaced with a computer. The CD spectrum of the 5 μM DNA aptamer was analyzed from 320 to 210 nm; the data gathered were the average of four scans at a scanning rate of 100 nm/min. The scan of the buffer recorded at room temperature was subtracted from the average scans for each DNA duplex. The data were collected in units of millidegrees versus wavelength and were normalized to the total DNA concentration.

2.3. SPR spectroscopic measurement

A home-built SPR biosensor [12,13] based on wavelength interrogation with a four-channel Teflon flow cell was used to monitor recognition interaction between aptamer and thrombin. The sensor chip was attached to the base of the prism, and optical contact was established using refractive index matching fluid (Cargille, USA). To obtain interaction kinetic measurements, the SPR was stabilized in carry buffer for 15 min. When the system reached a steady state, the sample was injected for 150 s (flow rate = 100 μl/min). Afterward, the carry buffer was used for washing and for diluting the sample.

2.4. Kinetic calculation

The affinity interactions between immobilized aptamer and thrombin in the solution were characterized by the association rate constant (k_{on}), dissociation rate constant (k_{off}), and equilibrium association constant (K_a). The sensitivity of the k_{on} and k_{off} in respond to variation of the binding conditions are the main study points based on the concept of "kinetic discrimination" [14]. The data were fitted using a simple 1:1 reversible interaction model, $A + B \leftrightarrow AB$, where A is the injected thrombin, B is the immobilized aptamer, and AB was the thrombin–aptamer complex formed during the reaction. In the SPR system, the signal R was proportional to the amount of [AB] and R_{max} was proportional to the initial [B]. A set of differential equations was applied to the data to find a solution for the three parameters k_{on}, k_{off}, and K_a, respectively. The experimental curves were recorded during the association and dissociation phases. The k_{off} was obtained from the dissociation phase using Eq. (1) first. Then k_{off} was substituted into Eq. (2) for obtaining k_{on} from the association phase. In a final step, K_a was calculated as the ratio of association and dissociation rate constants [15]:

$$R = R_0 e^{-k_{off} t} \quad (1)$$

$$R = \frac{k_{on}[A]R_{max}}{k_{on}[A] + k_{off}}[1 - e^{-((k_{on}[A]+k_{off})t)}] \quad (2)$$

3. Isothermal titration microcalorimetry

ITC experiments were performed using a MicroCal VP-ITC (MicroCal, Inc., Northampton, MA, USA) as reported previously [16]. The sample cell (1.44 ml) contained 0.45 μM DNA. 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 5 μM, 10 μl thrombin at intervals of 300 s were made with continuous stirring of the solution (at 310 r.p.m.) in the sample cell. Each injection generated a heat-burst curve (μcal s^{-1}) versus time (min). The area under each peak was determined by integration using Origin 5.0 software (Microcal, Inc.) to give the measure of the heat associated with the injection. The resulting associated temperatures were plotted against the molar ratio. The resulting experimental binding isotherm was corrected for the effect of titrating thrombin into binding buffer. The binding affinity and thermodynamic parameters of the binding process were obtained by fitting the integrated heats of binding the isotherm to the one-site binding model to give an association constant (K_a) and the binding enthalpy (ΔH). Consequently, the changes in Gibbs free energy (ΔG) and the changes in entropy (ΔS) can be calculated using Eqs. (3) and (4), where T is the reaction temperature (in K) and R is the gas constant (1.986 cal K^{-1} $smol^{-1}$).

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

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

4. Results and discussion

Conformational data of the aptamers before and after the binding with its target molecules provide essential information of the binding mechanism, we first examined the structure of the aptamers before binding by CD spectra. The CD spectrum of the pure 15-mer aptamer in Buffer I (the SELEX buffer for the 15-mer) displayed a negative band at 255 nm and a positive band at 245 nm and 290 nm (Fig. 1A), which is the characteristic of the G-quadruplex structure [8]. However, the CD spectrum of the 29-mer aptamer in