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A family of DNA aptamers with varied duplex region length that forms complexes with thrombin and prothrombin

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ABSTRACT

Structural properties determine binding affinities of DNA aptamers specific to thrombin. Our paper is the first to focus on a family of eight G-quadruplex-based aptamers with varied duplex region length (from two to eight base pairs). We have shown that the duplex, which is not the main binding domain, greatly influences the interaction with thrombin and prothrombin. Furthermore, the affinity of an aptamer to thrombin and prothrombin increases (respectively from 2.7×10^{-8} M to 5.6×10^{-10} M and from 1.8×10^{-5} M to 7.1×10^{-9} M) with an increase in the number of nucleotide pairs in the duplex region.

1. Introduction

Systematic evolution of ligands by exponential enrichment (SELEX) is a rapidly developing approach for in vitro selection of oligonucleic acids (aptamers) that bind to a specific target molecule; this procedure has been reviewed in a number of publications [1–5]. Aptamers are single-stranded oligonucleotide sequences with a length of a few tens of bases and high affinity for a specific target molecule. The affinity of aptamers is comparable with that of antibodies; however, aptamers are more thermally stable and maintain their structures over repeated cycles of denaturation/renaturation, and are easily modified by various chemical reactions. Of particular interest is directed improvement of aptamers selected by SELEX. A good example of effective improvements of sequences selected by SELEX is an aptamer specific to thrombin.

One of the first aptamers obtained by SELEX was specific to thrombin [6]. Thrombin is a multifunctional serine protease belonging to the chymotrypsin family. The first single-stranded DNA aptamer was isolated from a pool of $\sim 10^{13}$ oligonucleotide sequences and formed a complex with thrombin at a K_D of 25–200 nM. The aptamers were based on the 15-mer sequence, dGGTTGGTGTGGTTGG (15TBA) [6]. Holland et al. [7] demonstrated that an aptamer dramatically reduced thrombin inhibition and showed the potential for clinical applications of aptamers. According to X-ray and NMR data [8–11], eight guanines create two planar G-quartets ("chair" structure), named the G-quadruplex structure, bound by three loops: two short T–T loops and one T–G–T (Fig. 1A). Recently, improved DNA aptamers with additional oligonucleotide sequences at the 3′- and 5′-ends complementary to each other have been found to have high affinity for thrombin (K_D = 10–25 nM) [12,13]. One of the high-affinity aptamers based on G-quadruplex and duplex domains is RE31, which comprises 31 nucleotides [14]. It consists of the G-quadruplex (15 nucleotides identical to 15TBA) and a duplex (six complementary base pairs and two non-complementary base pair nucleotides). Clearly, further improvement will require a deep understanding of the interactions between the aptamers and

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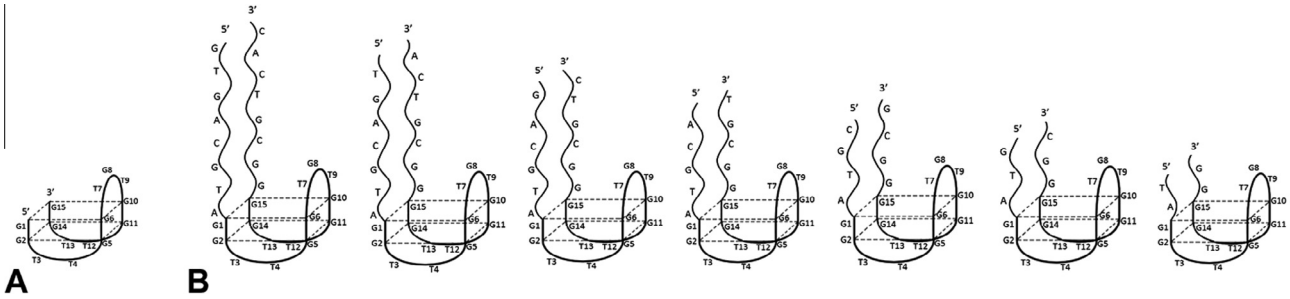

Fig. 1. Structures of 15TBA aptamer (A) and the proposed structures of aptamers RE31, RE29, RE27, RE25, RE23, RE21, and RE19 (left to right) (B).

thrombin. An important question to address is the structural and functional relationship between the number of base pairs that make up the duplex domain and affinity. Understanding this relationship will facilitate engineering of aptamer affinity.

The first attempts to address this issue were made in a study [15] examining a family of aptamers using circular dichroism (CD) spectroscopy. Structures of DNA aptamers confirmed by CD spectroscopy are shown in Fig. 1B. The range of DNA aptamers included 15TBA (no duplex), RE31 (eight base pairs, the maximum number of nucleotides in the duplex domain) aptamers, and a row of intermediate aptamers with a shortened duplex structure, namely RE29, RE27, RE25, RE23, RE21, and RE19. The stability of their complexes with thrombin has been evaluated using polyacrylamide gel electrophoresis. It has been shown that the presence of a duplex improves the affinity of aptamers to thrombin.

In this work, we decided to continue the study of this family of aptamers. Using surface plasmon resonance (SPR), we experimentally estimated how changes in the duplex domain affect the kinetics of the interaction between DNA aptamers and the target.

For these studies, the target was typically thrombin alone. In a cascade of proteolytic reactions, prothrombin, an inactive precursor, is converted to thrombin, which in turn actively participates in the blood clotting process by converting fibrinogen to fibrin and by activating platelets. However, any potential interactions of an aptamer with other members of this process have yet to be addressed. Only the studies conducted by Kretz et al. [16–18] raised the issue of how 15TBA (HD1) aptamer affects the binding of FXa–FVa complex (prothrombinase complex) with prothrombin during its conversion to thrombin. It has been shown that fluorescently tagged 15TBA (HD1) binds to both thrombin and prothrombin.

In this study, we undertook for the first time an integrated evaluation of kinetic constants and determined the affinity of prothrombin to the family of aptamers and compared the results with thrombin. We aimed to clarify the relationship between the structure of DNA aptamers and their affinity to protein molecules. The study is based on a family of DNA aptamers with duplex regions of variable length, which specifically interact with thrombin and prothrombin.

2. Materials and methods

2.1. Reagents

Experiments were performed using inorganic salts of reagent and chemically pure grades (Khimmed, Moscow, Russia), human α-thrombin, human prothrombin (Haematologic Technologies Inc., Vermont, USA), and the following oligonucleotides synthesized by the phosphoramidite method (Syntol, Moscow, Russia):

15TBA: biot-dGGTTGGTGTGGTTGG;
RE19: biot-dTAGGTTGGTGTGGTTGGGG;
RE21: biot-dGTAGGTTGGTGTGGTTGGGGC;
RE23: biot-dCGTAGGTTGGTGTGGTTGGGGCG;
RE25: biot-dACGTAGGTTGGTGTGGTTGGGGCGT;
RE27: biot-dGACGTAGGTTGGTGTGGTTGGGGCGTC;
RE29: biot-dTGACGTAGGTTGGTGTGGTTGGGGCGTCA;
RE31: biot-dGTGACGTAGGTTGGTGTGGTTGGGGCGTCAC.

2.2. Surface plasmon resonance

Measurements were performed using a Biacore X optical biosensor (GE Healthcare, Wisconsin, USA). A flow cell was filled with running buffer (20 mM HEPES–HCl, pH 7.2; 140 mM NaCl, 5 mM KCl), which was used for all measurements. The flow rate was 10 μL/min. Biotinylated aptamers (15TBA, RE19, RE21, RE23, RE25, RE27, RE29, RE31) were immobilized on streptavidin-coated SA sensor chips (because of the potential for formation of biotin–streptavidin complexes) according to the manufacturer's instructions. For kinetic experiments, eight different chips (each aptamer on one chip) were prepared. All aptamers were diluted to 2 μM in 10 mM HEPES–HCl (pH 7.4), 150 mM NaCl, and 0.005% Tween-20, and 60 μL of the aptamer solution were injected. Control cells were prepared in exactly the same manner except that 10 mM HEPES, 150 mM NaCl, 0.005% Tween-20 was injected instead of the aptamer.

During the experiments, for all aptamers, the concentration of thrombin was varied from 480 nM to 4 nM, and the concentration of prothrombin was varied from 1 μM to 13 nM. Thrombin (or prothrombin) was injected in a volume of 40 μL. After each interaction between an aptamer and thrombin (or prothrombin), the surface of the chip with the immobilized aptamer was regenerated with 20 μL of 2 M NaCl. Measurements were carried out in parallel with the control cell on the chip.

2.3. Data processing

The constants were calculated for each injection of thrombin or prothrombin. Errors of determination for each kinetic constant were provided from the analysis of experimental series with different concentrations of thrombin (or prothrombin). All results were obtained by subtracting the data for the control cell (the cell containing no immobilized analytes) to eliminate the contribution from the refractive index change caused by the injection of a new analyte. The data obtained using the Biacore X optical biosensor were processed by fitting of the binding profiles to a 1:1 (Langmuir) binding model using the BIAevaluation software.

When selecting ranges for the fitting, we used advanced features of the BIAevaluation software, in particular the option "split