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Probing the Force-Induced Dissociation of Aptamer-Protein Complexes

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Supporting Information

ABSTRACT: Aptamers are emerging as powerful synthetic bioreceptors for fundamental research, diagnostics, and therapeutics. For further advances, it is important to gain a better understanding of how aptamers interact with their targets. In this work, we have used magnetic force-induced dissociation experiments to study the dissociation process of two different aptamer–protein complexes, namely for hIgE and Ara h 1. The measurements show that both complexes exhibit dissociation with two distinct regimes: the dissociation rate depends weakly on the applied force at high forces but depends stronger on force at low forces. We attribute these observations to the existence of at least one intermediate state and at least two energy barriers in the aptamer–protein interaction. The measured spontaneous dissociation rate constants were validated with SPR using both Biacore and fiber optic technology. This work demonstrates the potential of the magnetic force-induced dissociation approach for an in-depth study of the dissociation kinetics of aptamer–protein bonds, which is not possible with SPR technologies. The results will help in the development and expansion of aptamers as bioaffinity probes.

Aptamers are short single-stranded DNA or RNA sequences typically selected using an in vitro method known as systematic evolution of ligands by exponential enrichment (SELEX),¹ which are capable of binding their targets with high specificity and affinity, rivaling the characteristics of antibodies. Although aptamers are becoming important in fields such as analytics,^{2–4} clinical diagnostics,^{5,6} and new therapeutics,^{7,8} the comprehension of the interaction between aptamers and their targets is still limited.

The specific interaction between aptamers and their target molecules has been successfully employed in the design of bioassays. The sensitivity and specificity of the bioassays rely on the affinity and strength of the aptamer–target interaction, which have been studied using several approaches. Surface plasmon resonance (SPR) has been the most common method to investigate biomolecular affinities.^{9,10} It is a label free, real-time detection technology that provides information on the interaction equilibrium and on the kinetics of the binding reaction. SPR has been applied for a wide range of applications that go from the study of antibody–protein¹¹ and DNA–protein interactions¹² to the selection of antibodies^{13,14} and the discovery of new drugs.^{15,16} However, despite these apparent advantages and being assumed as a standard reference technology to study biomolecular interaction processes, SPR gives limited insights into the mechanism of the interactions

due to the fact that it records only spontaneous association and dissociation processes.

An alternative way to study dissociation properties of molecular bonds is by force spectroscopy. This relies on the application of a force to a molecular bond and the measurement of its dissociation characteristics. In dynamic force spectroscopy (DFS),¹⁷ the studied molecular pair is first brought into contact to form the specific bond and then pulled apart at a specific loading rate until the bond is disrupted. Multiple approach and retract sequences provide a distribution of forces at which dissociation occurs. For each loading rate, a maximum probability for bond rupture can be found and these forces are used to calculate the statistically relevant dissociation rate, as well as the position along the reaction coordinate of the energy barrier (x_β). The main technique used in DFS experiments is atomic force microscopy (AFM),¹⁸ but DFS can also be performed with biomembrane force probes (BFP)^{19,20} and optical tweezers.²¹ However, because these techniques allow monitoring of only one interaction at a time and because molecular dissociation processes are of a stochastic nature, it is difficult and time-consuming to get good statistics on the experimental results. Moreover, the instrumentation

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Figure 1. Schematic overview of the technology platform consisting of (1) a microscope-camera system, (2) a sample holder, and (3) an electromagnet. The magnetic force-induced dissociation experiment is schematically shown on the right with the zoom-in area depicting the interaction between aptamer (purple) attached to magnetic particles (brown) and protein (pink) immobilized on a polystyrene surface.

involved is relatively complex, delicate, and expensive, with measurements being influenced by experimental conditions such as temperature stability, mechanical vibrations, air currents, acoustic, and electrical noise.²² Alternatively, magnetic tweezers^{22,24} can be used, which have the advantage that the same force can be applied on many magnetic particles at the same time, with each particle being bound to a solid phase by a single molecular bond. Measurements at different loading rates or at different constant forces can provide statistically relevant data in a single run.

To study aptamer–protein interactions, we used magnetic tweezers at a range of different constant forces as described by Jacob et al.²³ Superparamagnetic particles, functionalized with a receptor, were incubated with the respective ligand, which was physically adsorbed on a substrate. For each different magnetic pulling force, the number of particles detached from the surfaces was measured. From these experiments, the dissociation rate constant at zero force as well as the transition state distance in ligand–receptor complexes could be determined. The technology has already been applied to study the force-induced dissociation of antibody–protein pairs.²³ In this paper, we have applied the method to reveal the characteristics of force-induced aptamer–protein dissociation. Two different protein–aptamer complexes were chosen as a model system: (i) the complex formed between the human Immunoglobulin E (hIgE) protein and a well-known ssDNA aptamer selected and described in 1996 by Wiegand et al.²⁴ and (ii) a complex formed between the Ara h 1 protein, a major peanut allergen,²⁵ and a new aptamer selected for the development of novel techniques for detection of peanut allergens in food samples.²⁶

The dissociation constant values for both aptamer–protein complexes estimated with the magnetic force-induced dissociation approach were validated with a Biacore 3000 SPR analyzer, as a reference technique, and were compared to already available results obtained with a fiber-optic SPR platform.^{10,26} The results of this work show the suitability of magnetic force-induced dissociation to characterize aptamer–protein dissociation, revealing that this process undergoes at least one intermediate state and overcomes two activation barriers before final separation.

MATERIALS AND METHODS

Reagents and Biomolecules. All buffer reagents were supplied by Sigma-Aldrich (The Netherlands). All solutions were prepared using deionized water purified with a Milli-Q Simplicity 185 system (Millipore). Natural Ara h 1, extracted from light roasted peanut flour (purity < 95%), was purchased from Indoor Biotechnologies Limited. Human myeloma IgE (hIgE) kappa was supplied by Athens Research and Technology. Streptavidin-coated superparamagnetic particles of 2.8 μm diameter (Streptavidin Dynabeads M270) were purchased from Life Technologies (Norway). Biotinylated DNA aptamers against Ara h 1 (5'-TCGCACATTCGCTTCTACCGGGGGGGTCGAGCTGAGTGGATGCGAATCTGTGGGTGGGCGTAAGTCCGTGTGTGCGAA-3') and against hIgE (5'-GGGGCACGTTATCCGTCCCCTCCTA-GTGGCGTGGCC-3') were obtained from Integrated DNA technologies, Inc. (Belgium). The 37-mer aptamer against hIgE was extended with 24 thymidine bases at the 3' biotin binding site to give the aptamer maximum flexibility for binding with IgE protein.

Functionalization of Superparamagnetic Particles and Polystyrene Substrates. Biotinylated Ara h 1 aptamer and hIgE aptamer were coupled to streptavidin-coated Dynabeads M270. The particles were first washed with a buffer containing 10 mM Tris–HCl, 300 mM NaCl, and 1 mM EDTA at pH 7.5 and then incubated with 1 μM solution of the biotinylated aptamer for 30 min, being the particle concentration 5 mg/mL during the incubation. After modification, particles were washed twice with 150 mM PBS buffer (pH 7.4) containing 0.5% BSA and 0.01% Tween 20 and stored in the same buffer.

Polystyrene substrates 22 $\times$ 22 mm in size (Agar Scientific, U.K.) were cleaned with a nitrogen gas flow. Afterward, an open cell of 9 mm diameter and 0.12 mm depth was created on a polystyrene surface using a double-sided adhesive cell-dot (Secure-Seal imaging spacer, Sigma-Aldrich, The Netherlands). The two different ligands (Ara h 1 and hIgE protein) were immobilized by physical adsorption on the polystyrene surface enclosed within the cell. For that purpose, a 150 μL droplet of a protein solution prepared in 150 mM PBS buffer was incubated during 1 h at room temperature inside the cell area. Finally, all

modified polystyrene surfaces were blocked with 1% bovine serum albumin (BSA) solution also prepared in 150 mM PBS buffer.

Magnetic Force-Induced Dissociation Experiments.

The functionalized magnetic particles were diluted 5 times from stock concentration (10 mg/mL) for further application on the modified polystyrene surfaces. The cell was closed with a glass microscope coverslip, 15 × 15 mm in size, and the particles were incubated for 3 min on the treated surface. After this incubation period, the samples were inverted in order to remove unbound particles from the surface by gravitational forces. Subsequently, magnetic pulling forces of different magnitudes, all in the piconewton range, were applied on these samples (Figure 1).

Magnetic Force-Induced Dissociation Technology Platform. As shown in Figure 1, the magnetic force-induced dissociation technology setup consists of three different parts: (1) a microscope-camera system for imaging and recording, (2) a sample holder that supports the fluid cell in which the force-induced dissociation experiments take place (shown in Figure 1 on the right), and (3) an electromagnet to apply a constant mechanical pulling force in the piconewton regime.

This technology was previously described in detail in Jacob et al.²³ In short, the fluid cell with functionalized magnetic particles, bound to the top polystyrene surface, was placed on a sample holder lying on top of an electromagnet. This electromagnet consists of a copper wire coil around a soft iron core with a tapered end. The radius of the tip is 1 mm, and the distance between the binding surface and the tip is 300 μm. The magnetic field was controlled by the current going through the coil with the maximum current used in the experiments being 0.8 A. Because at this current a substantial amount of heat is generated in the coil, a water pump was used as a cooling system. This enabled continuous operation below 45 °C and prevented the coil from overheating. Moreover, to reduce the rise time of the current, a push–pull current controller that temporarily applies a higher voltage up to a maximum of 25 V was developed. In this way, the rise time of the current up to 95% was reduced to 4 ms. The same controller also allowed the application of time-dependent currents for demagnetization of the core. The magnet core was demagnetized after every force application. The magnetic forces were calibrated by a time-of-flight method, by which the magnitude of the force on the particles was determined by measuring the velocity of the particles in the applied magnetic field.²⁷ The applied force was calculated directly from the measured particle velocity and the Stokes equation for viscous drag. The advantage of this calibration method is that it does not rely on detailed information about the magnetic field, the magnetic field gradient, and the magnetic properties of the particles.

Imaging and Data Processing. The particles bound to the polystyrene surface were tracked with a Leica DM6000 microscope. Images were acquired at a 30 Hz frame rate by a RedLake MotionPro HS-3 speed camera triggered to the electromagnet through a function generator (Agilent, 33250A). The first frame at $t = 0$ captures the total number of particles bound on the surface before the application of the force. The detachment of the particles is observed from the second movie frame. By counting the particles that remain attached to the polystyrene surface across consecutive image frames, the dissociation rate curve for each applied force is obtained. The counting was done by a home-written MatLab (The MathWorks Inc.) program, which gives the number of particles that

are still left in each frame as an output. The fitting of the dissociation curves was performed using Origin software (The OriginLab Corporation).

Dependence of the Dissociation Rate Constant of Biomolecular Complexes on the Applied Force. The force dependence of the dissociation of molecular bonds has been extensively described in literature.^{17,20,28,29} In accordance with the Bell and Evans model,^{17,28} the dissociation rate constant of a molecular bond at zero force, $k_{\text{off}}(0)$ (s^{-1}), can be related to the change in free energy of the transition state, $E_b(0)$, by the following expression:

$$k_{\text{off}}(0) = \nu \cdot \exp[-E_b(0)/k_b T] \quad (1)$$

where ν is the attempt frequency that depends on the molecular details of the interaction, k_b is the Boltzmann constant, and T is the temperature. When a transitional pulling force, F (pN), is applied, the energy needed to overcome the transition state is lowered by the work done along the reaction coordinate upon dissociation: $W = -Fx_b$, where x_b (nm) is the distance between the minimum and the maximum of the energy barrier (i.e., the distance to which the molecules must be separated in order to break the bond between them). Therefore, the dissociation rate of a molecular bond changes exponentially with the applied force:

$$\begin{aligned} k_{\text{off}}(F) &= \nu \cdot \exp[-(E_b(0) - Fx_b)/k_b T] \\ &= k_{\text{off}}(0) \cdot \exp[Fx_b/k_b T] \end{aligned} \quad (2)$$

Thus, by varying the force applied to break the bond between ligand and receptor, it is possible to extrapolate the dissociation constant to zero force, $k_{\text{off}}(0)$, as the logarithm of $k_{\text{off}}(F)$ increases linearly with the increase in force, F :

$$\ln k_{\text{off}}(F) = \ln k_{\text{off}}(0) + Fx_b/k_b T \quad (3)$$

From this expression the transition-state distance x_b can also be obtained. With dependence on the different populations of bound particles in the samples (e.g., specifically vs nonspecifically bound particles, or single bond vs multiple bond particles), a mono- or a multicomponent dissociation pattern is displayed in the dissociation curves. Accordingly, the data analysis should be performed with a monoexponential or a multiexponential model.²³

Surface Plasmon Resonance Experiments on Biacore.

Surface plasmon resonance (SPR) experiments were conducted on a Biacore 3000 analytical system (Biacore AB, Sweden) using Biacore sensor chips CM5 consisting of a gold-coated glass slide modified with a carboxymethyl dextran layer. For the two aptamers, different optimum experimental conditions were found. In the case of Ara h 1 aptamer, streptavidin was first covalently coupled (1800 RU) to the chip surface through EDC/NHS chemistry.³⁰ Unbound streptavidin was removed from the sensor surface using a solution of 1 M NaCl and 50 mM NaOH. Consequently, a 50 nM solution of biotinylated aptamer against Ara h 1 protein prepared in HBS-EP buffer [10 mM HEPES pH 7.4, 450 mM NaCl, 3 mM EDTA, 0.005% (v/v) polysorbate 20] was injected up to 100 RU. For hIgE aptamer, streptavidin was coupled to the chip up to 300 RU followed by surface stabilization with 1 M NaCl and 50 mM NaOH solution. The biotinylated aptamer (5 nM), also prepared in HBS-EP buffer, was injected until 15 RU were reached. After coupling the aptamers, the chip was thoroughly washed with a 1 M NaCl (pH 12) to remove any nonspecific interactions. The two target proteins were injected in different

concentrations, ranging from 200 to 550 nM for Ara h 1 protein and from 4 to 64 nM for hIgE protein. The 1 M NaCl solution was also used to regenerate the chip between consecutive analyses. A sensorgram was obtained for each concentration whereas a cell activated and deactivated was used as a reference channel in all the experiments. To determine the binding kinetics, data were analyzed using the Biacore evaluation software 4.1 (Biacore AB, Sweden). Association and dissociation were fitted globally by a 1:1 Langmuir based model. The obtained curves and fitted data are shown in Figures S5 and S6 of the Supporting Information.

RESULTS AND DISCUSSION

Fitting Model for the Magnetic Force-Induced Dissociation of Aptamer–Protein Bonds. To establish the experimental conditions and evaluate the appropriate fitting model for studying the magnetic force-induced dissociation of DNA aptamers from proteins, we used the complex formed between the hIgE protein and its ssDNA aptamer as a model system. Since its discovery by Wiegand et al.,²⁴ this aptamer has been extensively used due to its high affinity and specificity toward hIgE, a protein that plays an important role in many allergic reactions. The hIgE aptamer has been successfully implemented in different transducer systems for developing biosensors, which proved to be a valuable alternative to immunosensors.^{10,31}

As explained in Magnetic Force-Induced Dissociation Experiments, superparamagnetic particles were functionalized with the hIgE aptamer and incubated with polystyrene surfaces functionalized with the corresponding ligand, hIgE protein. Because the dissociation of a molecular bond is a stochastic process determined by thermal fluctuations, it is important to average the individual behavior of many single-molecule dissociations in order to be able to extrapolate dissociation constant values.³² Therefore, the concentration of protein to be immobilized on the polystyrene surface is an important parameter to optimize. If the density of a protein on the surface is too high, multiple bonds may occur between the aptamer-coated particles and the surface, while on the other hand, if the concentration is too low, the number of single-bonds formed will be insignificant for statistical considerations. Thus, different concentrations of hIgE protein, ranging from 0.1 to 100 nM, were tested on the polystyrene slides (Figure 2). As a negative control, magnetic particles functionalized with hIgE aptamer were also incubated with a polystyrene slide coated only with 1% BSA, since BSA is used in experiments as a final blocking agent for all polystyrene surfaces, or with 70 nM of IgG protein. These negative controls gave typical bound particle numbers of less than 20 particles. As can be seen in Figure 2, binding of functionalized magnetic particles to the polystyrene surfaces occurs significantly when surfaces are coated with hIgE protein. This shows that there is hardly any nonspecific adsorption of magnetic particles and that their binding to the surface is mainly due to the interaction between the aptamer and its ligand.

To test whether these specific bonds between aptamer-coated particles and the protein-coated surface are of a single or multiple nature, we studied the behavior of the dissociation curves as a function of protein concentration on the surface. Therefore, dissociation curves were recorded for surfaces coated with different hIgE protein concentrations in the nanomolar range (10–1000 nM) at a constant applied force (5 pN) (see the Supporting Information). The curves show two

Figure 2. Images of magnetic particles as recorded by the camera. The particles are coated with hIgE aptamer and are bound to polystyrene surfaces modified with (A) 1% BSA, (B) 70 nM of IgG, and (C–F) different concentrations of hIgE protein (0.1, 1, 10, and 100 nM). The imaged area is about $250 \times 200 \mu\text{m}$.

regimes, representing a fraction of fast dissociation and a fraction of slow dissociation, where the fraction of slow dissociation strongly increases with increasing hIgE concentration. We attribute the fast fraction to particles bound by single specific bonds and the slow fraction to strong multiply bound particles. The application of a double exponent fitting model shows that the population of multiple bonds increases with the concentration of hIgE protein present on the surface (Figure S3 of the Supporting Information), while the dissociation rate for the disruption of these bonds was decreasing (Figure S4 of the Supporting Information). From these results, we can conclude that multiply bound particles are present in these experiments. To investigate if we are still able to deduce reliable dissociation rates for the fastly dissociating fraction, we have applied a single-exponential model to the data. The model contains a term representative for the single molecular bonds (N_s , k_{off}^s) and an offset term $[1 - N_s(0)/N_{\text{tot}}(0)]$ that is related to the particles bound by multiple bonds:

$$n(t) = N_{\text{tot}}(t)/N_{\text{tot}}(0) = N_s(0)/N_{\text{tot}}(0) \cdot \exp[-k_{\text{off}}^s(F) \times t] + [1 - N_s(0)/N_{\text{tot}}(0)] \quad (4)$$

where $N_{\text{tot}}(t)$ is the total number of particles bound to the surface as a function of time t , $N_s(0)$ is the number of particles bound to the surface through a single specific bond at time $t = 0$, t is the time during which the magnetic force is applied, and $k_{\text{off}}^s(F)$ is the dissociation constant corresponding to the single specific bonds. The parameter $n(t)$ represents the ratio between

Figure 3. Results of the single-exponential model fit to the hIgE–aptamer dissociation data. Panel (A) shows the dissociation rate k_{off} and panel (B) shows the fraction of single specific bonds at the start of the measurement $N_s(0)/N_{\text{tot}}(0)$. The values were determined by fitting eq 4 (single exponential with offset) to the data of Figure S2 of the Supporting Information; the fits are shown in Figure S5 of the Supporting Information.

Figure 4. (A) Curves representing dissociation of hlgE aptamer-functionalized magnetic particles from a hlgE protein-coated surface as a function of time. The curves are averages of at least two replicas. For each applied pulling force, the curves were fitted using eq 4. The polystyrene surfaces were incubated with 100 nM hlgE. (B) The fitted single-molecular dissociation rate of hlgE–aptamer bonds as a function of the applied force. The curve shows two distinct regimes, which can be extrapolated to zero force to obtain the natural dissociation rate constant. The dashed line indicates that the sketched line is an interpretation with uncertainty, since the linear fitting is done using only two low-force data points.

the total number of bound particles at time t divided by the total number of bound particles at $t = 0$.

We found that the single-exponential model gives consistent values for the dissociation rate constant of $0.18 \pm 0.02 \text{ s}^{-1}$ in the hlgE concentration range between 30 and 350 nM (Figure 3A). In that concentration range, the fraction of single specific bonds $[N_s(0)/N_{\text{tot}}(0)]$ varied between 90% and 30% (Figure 3B).

The magnetic force-induced experiments were performed at 5 pN force. The arrow indicates the range wherein consistent values for k_{off} are determined, which we can attribute to the dissociation of single specific bonds.

From these results, a concentration of 100 nM hlgE protein during incubation on the polystyrene surface was chosen as a suitable concentration for the magnetic force-induced dissociation experiments. At this concentration (i) the amount of bound particles is well above background and statistically relevant (see Figure 2), (ii) single specific bonds are dominating over multiple bonds (see Figure S3 of the Supporting Information), and (iii) concentration-independent dissociation rates are extracted (see Figure 3), which gives confidence that single-bond dissociation properties are being measured.

Study of the hlgE–Aptamer Complex. Subsequently, magnetic force-induced dissociation experiments for the hlgE aptamer–protein complex were performed by applying different magnetic pulling forces, ranging from 2.5 to 20 pN, to the particles bound to the functionalized polystyrene surfaces. For each applied force, at least two different samples were analyzed

resulting in a dissociation curve for each measurement. It is important to note that the weakest link in the applied polystyrene–protein–aptamer–particle system is the molecular bond formed between the protein and the aptamer, so this is the site where the complex will dissociate upon the application of a magnetic pulling force. This assumption was already proven when the same assay was performed with a protein–antibody complex that has a bond comparable or even stronger than the one formed between aptamer and protein.²³ In addition, it has been described in literature that proteins physically adsorb to polystyrene surfaces through stronger interactions compared to protein–ligand bonds.³³

The dissociation curves obtained when applying different magnetic pulling forces to the hlgE aptamer–protein complex (Figure 4A) were fitted using eq 4. From this equation, the dissociation rate for the specific interactions $[k_{\text{off}}(F)_s]$, as well as the population of particles specifically bound in each experiment at time $t = 0$ $[N_s(0)/N_{\text{tot}}(0)]$ were obtained. The extracted values of $N_s(0)/N_{\text{tot}}(0)$ varied between 0.4 and 0.7, without showing a clear dependence on force.

The extracted dissociation rates of the specific bonds were plotted as a function of the applied force on a logarithmic scale, according to eq 3 (Dependence of the Dissociation Rate Constant of Biomolecular Complexes on the Applied Force). The equation predicts a linear relationship between the applied force, F , and the logarithm of the dissociation constant. Interestingly, for the dissociation constant of the hlgE–aptamer complex, we found that low-force data points lie below the extrapolation line of the high-force data points, suggesting the

presence of two distinct linear regions (Figure 4B). An explanation for this behavior might be the existence of more than one energy barrier for dissociation along the reaction coordinate. The presence of a cascade of energy barriers is generally associated with widely distributed atomic-scale interactions (i.e., specific noncovalent interactions).¹⁷ When an external force is applied, the hierarchy of the energy barriers could change, as sketched in Figure 5.

Figure 5. Conceptual energy profile for an aptamer–protein dissociation process through an intermediate state with two energy barriers (blue line). Tilted energy landscape under an applied low force (f_1), where the outer barrier remains as the dominant one (green dashed line), and tilted energy landscape under an applied strong force (f_2), where the outer energy barrier is driven below the inner one, which emerges to dominate kinetics (red dashed line). The x -coordinate denotes the molecular coordinate or bond distance. $x_{\beta 1}$ and $x_{\beta 2}$ are the transition state distances.

The natural dissociation of a complex in the absence of external forces resembles the dissociation in the regime of low

magnetic pulling forces, with the outer barrier being the leading one in governing the kinetics of the dissociation. However, as seen in the graph, if the applied force is large enough, outer barriers can be driven below inner barriers, making the inner barrier become the dominant impedance for the complete unbinding of the complex. Consequently, a sequence of linear regimes may appear when plotting the logarithm of the dissociation rate versus the force, assuming that all the barriers lie along a single, one-dimensional escape path.³⁴ Therefore, the two regimes in Figure 4B suggest that the dissociation process of the hIgE aptamer from the hIgE protein may occur through at least two activation energy barriers and undergoes at least one intermediate state. This interpretation is consistent with the existence of different folding intermediates that the aptamer undergoes when interacting with proteins and that facilitate the target binding. These folding intermediates are the result of the presence of different binding pockets in the aptamer's structure,^{35,36} defined by diverse recognition motifs and structural elements in the DNA that are formed by specific oligonucleotide sequences, as well as of the several kinds of interactions that occur between the aptamer and the target protein, such as electrostatic and ionic, hydrogen bonds, and van der Waals forces.

When the dissociation constant is extrapolated to zero force, $k_{\text{off}}(0)$, along the two linear segments in Figure 4B, two values are determined for the hIgE aptamer–protein complex: one from the low-force segment corresponding to the outer energy barrier in the energy landscape, being $(4 \pm 2) \cdot 10^{-3} \text{ s}^{-1}$ and one derived from the high-force segment corresponding to the inner energy barrier in the energy profile, being $0.07 (\pm 0.02) \text{ s}^{-1}$. The transition state distances, x_b , were found to be $22 \pm 3 \text{ \AA}$ for the outer barrier and $2.2 \pm 0.9 \text{ \AA}$ for the inner barrier.

Study of the Ara h 1–Aptamer Complex. The force-induced dissociation properties between protein and DNA aptamer complexes were also studied on the protein–aptamer pair formed between Ara h 1 and a recently selected aptamer against this protein.²⁶ This aptamer was shown to be a promising alternative to antibodies for application in new technologies for the detection of allergens in food samples. As in the previous experiments, nonspecific adsorption of magnetic particles to polystyrene slides was first tested, which similarly resulted in absence of binding (less than 20 particles) when slides were coated with 1% BSA or a nonspecific protein (IgE). In specific binding experiments, the Ara h 1 protein was

Figure 6. (A) Curves representing dissociation of Ara h 1 aptamer-functionalized magnetic particles from an Ara h 1 protein-coated surface as a function of time. The curves are averages of at least three replicas. For each applied pulling force, the curves were fitted using eq 4. The polystyrene surfaces were incubated with 100 nM Ara h 1. (B) The fitted single-molecular dissociation rate of Ara h 1–aptamer bonds as a function of the applied force. The curve shows two distinct regimes, which can be extrapolated to zero force to obtain the natural dissociation rate constant. The dashed line indicates that the sketched line is an interpretation with uncertainty, since the linear fitting is done using only two low-force data points.

Table 1. Dissociation Constants of the hIgE Aptamer–Protein Complex and the Ara h 1 Aptamer–Protein Complex Determined by Force-Induced Dissociation Experiments, FO-SPR and Biacore^a

$k_{\text{off}}(0)$ hIgE protein–aptamer (s^{-1})			$k_{\text{off}}(0)$ Ara h 1 protein–aptamer (s^{-1})		
magnetic force-induced dissociation	FO-SPR	Biacore	magnetic force-induced dissociation	FO-SPR	Biacore
low forces: $(4 \pm 2) \cdot 10^{-3}$ high forces: 0.07 ± 0.02	$(2.1 \pm 0.2) \times 10^{-3}$	$(7.5 \pm 0.8) \times 10^{-4}$	low forces: $\sim 4 \times 10^{-2}$ high forces: 0.18 ± 0.03	$(6.7 \pm 0.1) \times 10^{-2}$	$(3.9 \pm 0.1) \times 10^{-2}$

^aThe obtained magnetic force-induced dissociation constants at low forces are indicative values since they are calculated from a linear fit of the dissociation constant values obtained at only two measured forces (see Figures 4 and 6). In the case of the Ara h 1 protein–aptamer complex, the available data at low forces is not sufficient to extract an error.

immobilized on the polystyrene surfaces at a concentration of 100 nM.

Next, a range of magnetic pulling forces, from 2.5 to 20 pN, was applied to the bound particles, resulting in exponential dissociation curves (Figure 6A). Fitting the data with the already established exponential model (eq 4) yields a force-dependence of the dissociation rate with two regimes (see Figure 6B): the dissociation rate depends weakly on the applied force at high forces (10–20 pN) and the lowest-force data points (5–10 pN) clearly deviate from the high-force linear fit. This may again indicate that two energy barriers separate the native form from the unbound state of the molecular system.

The dissociation constants extrapolated to zero force for the Ara h 1 protein–aptamer complex were $\sim 4 \times 10^{-2} \text{ s}^{-1}$ at low applied forces (cf. the outer barrier) and $0.18 \pm 0.03 \text{ s}^{-1}$ at high applied forces (cf. the inner barrier). The values for x_b were found to be about 9 Å for the outer energy barrier and 2.2 ± 0.4 Å for the inner energy barrier. Interestingly, the value obtained for the inner-barrier transition state distance is the same as the one obtained for the inner barrier of the hIgE complex. This may suggest that the inner barrier is related to a nonspecific interaction between the DNA aptamer and the protein; to test this hypothesis clearly more DNA–aptamer systems should be studied.

3.4. Validation of Dissociation Constants by SPR Experiments. To validate our results, dissociation constant values for each of the protein–aptamer complexes were also determined by Biacore SPR experiments. Moreover, the values obtained with magnetic force-induced dissociation were compared to dissociation constants previously obtained through fiber optic-SPR (FO-SPR) measurements and reported in literature^{10,26} (Table 1).

The SPR values, from both FO-SPR and Biacore experiments, obtained for the two aptamer–protein complexes were in the same range as the extrapolated force-induced dissociation constants obtained at low applied magnetic forces, with the closest agreement observed for the Ara h 1 system. This gives confidence to the extracted values at low applied forces and indicates that at zero force, similar molecular processes are being probed by these three analysis methods.

CONCLUSION

Molecular dissociation stimulated by the application of magnetic forces was studied for the first time on bonds established between DNA aptamers and proteins. Two different aptamer–protein complexes were investigated, namely for hIgE and Ara h 1. Interestingly, we found for both molecular systems that the obtained low-force dissociation constants lie clearly below the extrapolation line of the high-force dissociation constants (see Figures 4B and 6B) (i.e., the dissociation rate depends weakly on the applied force at high forces and depends

stronger on force at low forces). Dissociation constants were measured using three different approaches (magnetic force, planar SPR, and fiber-optic SPR). The data show that the low-force dissociation rates are close to the natural dissociation measured by the SPR studies. We hypothesize that the higher values of the dissociation constants obtained by extrapolating the high-force data to zero force [$k_{\text{off}}(0) = 0.071 \text{ s}^{-1}$ for hIgE and 0.18 s^{-1} for Ara h 1] reflect an inner energy barrier of the protein–aptamer dissociation pathway. The results point to a dissociation pathway of aptamer–protein complexes that goes through at least one intermediate state and has to overcome at least two energy barriers before the bond is completely disrupted. The inner barrier may be due to nonspecific interactions of the complex (e.g., the electrostatic forces occurring between charged functional groups of the protein and the sugar–phosphate backbone of DNA^{37,38}), as higher values of the dissociation rate constant are usually observed for nonspecific bonds. The fact that the same transition state distance value (2.2 Å) was determined for both aptamer–protein complexes when high forces were applied, is in agreement with this hypothesis. This work demonstrates the potential of magnetic force-induced dissociation for an in-depth study of the dissociation kinetics of the biomolecular bonds formed between aptamers and proteins, which is not possible with technologies that record only the spontaneous dissociation rate, such as SPR. Moreover, the results are in harmony with previous work by Ge et al.,³⁹ performed with thrombin and a corresponding bivalent aptamer. With the use of AFM, it was found that during the unbinding from the thrombin of both sequences present in the bivalent aptamer, one intermediate state with two activation barriers was encountered. Similarly, inner barriers were reported with short transition state distances and high dissociation rates, in contrast with outer barriers with much larger distances to the transition state and lower unfolding rates. Comparable transition state distance values were found for the inner activation barrier (1.1–2.2 Å) but remarkably different ones for the outer activation barrier, which further supports our hypothesis. In addition, single-molecule force spectroscopy applied by Greenleaf et al.⁴⁰ to study hierarchical folding in single riboswitch aptamers also displayed that three states are seen in the overall unfolding process: the folded, intermediate, and unfolded states; however, values of k_{off} and x_b for the individual barriers were not reported.

Thus, both previously reported work and the present study suggest that aptamers dissociate from their target protein following a multistep dissociation process that would involve more than one energy barrier. Alternatively, in a recent study, Fridel et al.⁴¹ introduced a different interpretation of the two linear trends observed in force spectra obtained by dynamic force spectroscopy, attributing the trends to the reformation of

single bonds (rebinding) at slow loading rates. They suggested that the two-barrier interpretation could be a misconception, because previous publications presenting nonlinear force spectra and using a two-barrier interpretation reported a distance to the inner barrier that was unrealistically small, namely less than one 1 Å. However, the transition state distances deduced in this work are very realistic (2.2 Å) and give confidence that the two-barrier interpretation may indeed be correct for the constant-force experiments reported in this paper.

To further substantiate the conclusions, additional force-induced dissociation experiments should focus on acquiring more data (in order to derive more accurate values of the dissociation rates) and on studying other aptamer–protein systems. The results will help to further understand the interactions between aptamers and target proteins, which is important from a theoretical and a practical point of view, because the kinetics and specificity of aptamer–protein interactions are very important for diagnostic and therapeutic applications.

■ ASSOCIATED CONTENT

■ Supporting Information

Additional information as reported in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

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