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Probing interactions within the synaptic DNA-SfiI complex by AFM force spectroscopy

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Abstract

SfiI belongs to a family of restriction enzymes that function as tetramers binding two recognition regions for the DNA cleavage reaction. SfiI protein is an attractive and convenient model for studying synaptic complexes between DNA and proteins capable of site specific binding. SfiI enzymatic action has been very well characterized. However, properties of the complex prior to the cleavage reaction are not clear yet. We applied AFM single molecule force spectroscopy to analyze the strength of interactions within the SfiI - DNA complex. In these experiments, the stability of the synaptic complex formed by the enzyme and two DNA duplexes was probed in a series of approach-retraction cycles. In order to do this, one duplex was tethered to the surface and another one to the AFM probe. The complex was formed by the protein present in the solution. An alternative setup in which the protein was anchored to the surface allowed us to probe the stability of the complex formed with one duplex only in the approach-retraction experiments, with the duplex immobilized at the AFM tip. Both types of complexes are characterized by similar rupture forces. The stability of the complex was determined by measuring the dependence of rupture forces on force loading rates (dynamic force spectroscopy - DFS). The DFS data suggest that the dissociation reaction of SfiI-DNA complex has a single energy barrier along the dissociation path. Dynamic force spectroscopy was also instrumental in revealing the role of the 5 base pair spacer region within the palindromic recognition site on DNA-SfiI complex stability. The data show that, although the change of nonspecific sequence does not alter the position of activation barrier, it significantly changes values of the off rates.

Keywords

synaptic complex; force spectroscopy; protein-DNA interactions; AFM; site-specific recognition

Introduction

SfiI as a member of subgroup IIF restriction enzymes functions as a homotetramer.¹ It recognizes and binds specifically two DNA duplexes containing 13 base pair recognition sites 5'-GGCCNNNN↓NGGCC-3' (N is any base; arrow indicates the cleavage position), in which two tetranucleotide specific sequences are separated by a non-specific 5-base pair region. Interestingly, although the sequence of this internal 5 base pair spacer is not critical for the site specific recognition, it modulates the rate of the cleavage reaction.² Magnesium is required as a cofactor for SfiI to cleave DNA, however in the presence of Ca²⁺ ions, instead of Mg²⁺, SfiI forms a stable complex without DNA cleavage.³ This makes SfiI a very convenient object for

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studying the formation of synaptic complex, and its characteristic features such as site specific recognition and binding.

SfiI enzymatic activity has been very well characterized by Halford and coworkers.⁴ However, structural data for this system were not available until recently. We⁵ used AFM to resolve the arrangement of the DNA duplexes within the SfiI-DNA complex. This study showed that the two DNA recognition regions remain almost straight and are crossed at an angle of 60° with respect to each other. Simultaneously published crystallographic data for the SfiI-DNA complex⁶ were in a perfect coincidence with the AFM results. According to crystallographic data, each SfiI monomer binds a 4 bp (-GGCC-) sequence, so that SfiI dimeric unit can bind only one duplex with the entire 13 bp recognition site. SfiI protein has been shown to exist as a tetramer in solution.⁷ The SfiI tetramer, therefore, can form either a synaptic complex with two duplexes or a pre-synaptic complex where only one duplex is bound to the protein. The central part of the entire DNA binding region (-NNNN[↓]N-) is not essential for binding specificity, as the protein has no direct contacts with the bases inside the spacer. Naturally occurring sequences are cleaved by SfiI at different rates.⁷ The nature of this effect is still not fully understood, however, the flexibility of the spacer was discussed as the most probable cause of the effect.^{2; 6}

Despite the fact that SfiI enzyme is well characterized functionally,^{1; 4; 7; 8; 9; 10; 11; 12} there are no data available about stability of the complex and kinetics of the complex dissociation. Availability of these data is important for understanding the mechanisms of the complex formation and the way the recognition process proceeds. In this study, we applied single molecule force spectroscopy to further characterize properties of the SfiI-DNA complex. Single molecule force spectroscopy has recently evolved into a powerful method to measure strength of intermolecular interactions on a single molecule level.^{13; 14; 15; 16} Also, the information obtained by the dynamic force spectroscopy (DFS), can be related to thermodynamic and kinetic parameters of the donor-acceptor system measured in bulk.^{17; 18; 19; 20} Such experiments often require covalent attachment of the target molecules, and we have recently developed and tested the surface chemistry which allows simple and reliable anchoring of target molecules with free thiol groups to a surface.²¹

By using single molecule force spectroscopy, we measured the stability of the synaptic SfiI-DNA complex. We also used DFS to reveal the energy landscape for the SfiI-DNA complex dissociation, which is not accessible by any other technique. The data of DFS showed that the dissociation reaction of the SfiI-DNA complex is defined by a single energy barrier. The dissociation rate values were found to be quite high, suggesting a dynamic nature of complexes for the studied DNA substrates. The contribution of the nonspecific spacer in the DNA recognition site to the stability of the complex was studied, and the role of this sequence in the complex formation is discussed.

Results

Experimental design

Double stranded 40 base pair oligonucleotides were designed to contain a 13 bp recognition sequence (5'-GGCCNNNNNGGCC-3') near 3' end. The thiol group modification at the 5' end provided a means for covalent anchoring of the duplex to surfaces. The thiol group was attached via flexible single-stranded DNA, using either dT₁₀ or dT₅₀ linker to reduce potential stereochemical complications for the formation of the complex of SfiI with tethered DNA duplex. The mica and the AFM tip surfaces were functionalized using the maleimide surface immobilization chemistry (see Methods section).²¹

Figure 1 schematically illustrates the experimental setup used for studying synaptic complexes. With no SfiI protein present in the solution, the probing of interactions between the tip and the surface produced zero rupture events and only short-range adhesion forces were observed (Fig. 1, a). Adding the SfiI protein resulted in the formation of a complex between protein and DNA duplexes after the tip approached the surface (Fig. 1, b). The complex is probed by applying a pulling force to the formed complex (Fig. 1, c). Note that due to the symmetry of the complex, both duplexes can dissociate upon applying the force. Only one option (dissociation of the left duplex) is shown in the figure for simplicity.

Figure 2 shows our approach for probing the stability of the pre-synaptic SfiI-DNA complex. SfiI protein was covalently anchored to the mica surface via –SH group of cysteine residue of SfiI. There is a unique cysteine residue near the C-terminus of the protein (230th out of 269), and according to the crystallographic data, this part of the protein is not involved in the recognition site of the protein.⁶ The interactions between DNA and the protein in the pre-synaptic complex were probed with DNA oligonucleotide attached to the AFM tip (Fig. 2, a).

Force spectroscopy study of synaptic SfiI-DNA complex

A typical force-distance curve obtained using the experimental setup for studying the synaptic complex schematically represented by Figure 1, a-c is shown in Figure 3. An adhesive peak at the beginning of the force curve (section 1 of the force curve) is accompanied by a force extension curve (section 2) corresponding to the stretching of polymer linkers²², followed by a rupture event (section 3).²¹ We interpret the magnitude of this force change as the force required to break the interactions between DNA and SfiI protein within the complex. This assumption was supported by control experiments in which the pulling was performed in a large excess of free duplex in solution. Binding of free duplexes to SfiI prevents the formation of the complex during approach of the tip to the surface and thus no rupture events are detected. Also, the rupture effect is not observed if EDTA is added to the protein solution due to removal of divalent cations which are critical for the SfiI-DNA complex formation. These control experiments provide strong evidence to our interpretation of a rupture event as breaking specific interactions between SfiI and DNA.

The forces required to break synaptic complex were systematically analyzed and compiled in a statistical histogram. The inset of Figure 3 shows such a histogram for the data obtained from analysis of complex rupture forces at 150 nm/s pulling velocity (9.5 nN/s apparent loading rate). The Gaussian fit to the distribution provides the most probable rupture force 45 ± 3 pN.

Additional evidence to the force spectroscopy data interpretation as the force-induced dissociation of the SfiI-DNA complex comes from the measurements of the linker extension from zero distance to the position of the complex rupture in a force-distance curve. As it is shown in Figure 3, contour length of the extended linker determined from the approximation of the elastic part by WLC model (see Methods section) is 36.5 nm. This value is in a good agreement with expected extension of the linkers for the system (ca. 36 nm). The expected rupture length was estimated by adding together lengths of maleimide silatrane linker containing 5 polyethylene glycol monomeric units²¹ in all-trans conformation (3.3 nm), size of the SfiI itself (6.8 nm),⁶ extension of the oligoT₁₀ spacer and the part of oligonucleotides which is not complexed with the protein assuming 0.34 nm distance per base pair.

We also performed experiments with the linkers of varied lengths. The results of measurements of the rupture length values performed over 500 of various rupture events for different linkers are shown in Figures 4 a-c. The most probable rupture lengths are the maxima of the distribution obtained as mean values from Gaussian fit. The rupture lengths are equal to 39, 53 and 71 nm for 10 nucleotides long polyT (Fig. 4, a), 50 nucleotides long polyT (Fig. 4, b) and polyethylene glycol (Fig. 4, c) linkers, respectively. The polyethylene glycol linkers were used to attach the

oligonucleotide to the AFM tip. These numbers are in good agreement with the expected values which are equal to 36, 50 and 61 for (dT)₁₀, (dT)₅₀ and PEG linkers respectively, calculated as described above. The increased width of the distribution for the experiments with long PEG linker (histogram c in Fig. 4) is attributed to the length heterogeneity of commercially available polyethylene glycol polymer (MW=3400 g/mol) ranging between 20 nm and 30 nm.²³

Force spectroscopy study of SfiI-DNA complex, SfiI immobilization approach

An alternative strategy depicted in Figure 2a was employed to investigate the pre-synaptic SfiI-DNA complex. In this approach, the enzyme was covalently attached to maleimide functionalized mica surface via unique cysteine moiety of the protein, and the interactions were probed with the oligonucleotide anchored to the AFM tip. A representative force-distance curve obtained using this experimental setup is shown in Figure 5. This curve is similar to the one obtained for the synaptic complex (Fig. 3) and has characteristic rupture event illustrating that the complex formation between single DNA duplex and the enzyme is studied. Only force curves with single rupture events as the one shown in Figure 5 were analyzed. The results of the rupture force measurements for a large set of such curves are summarized as a histogram in the inset of Figure 5. The most probable rupture force for the dissociation of the DNA-SfiI complex is 80 ± 5 pN at 150 nm/s pulling velocity (9.5 nN/s apparent loading rate) that is almost two times higher than the rupture force value obtained for the synaptic complex (Fig. 3).

We also carried out experiments in which the probing of immobilized SfiI by the tip terminated with the DNA duplex was performed in the presence of DNA duplex containing the SfiI binding site in the solution. The motivation for these experiments was that synaptic complex in addition to the pre-synaptic one can be formed and probed as well. Free DNA duplex can bind to the tethered enzyme occupying one of the binding sites, so only one DNA binding site remains available for the probing. Schematically this possibility is illustrated in Fig 2d-e. The experimental data obtained at 150nm/s pulling velocity (~ 6.5 nN/s apparent loading rate) are shown as histograms in Figure 6a. Indeed, two distinct peaks with the mean values 33 and 63 pN are clearly resolved on these distributions suggesting the formation of two types of complexes probed by the AFM pulling experiments. The extensions of the linker were measured from the force curves and the results are summarized in Figure 6b. The experimental value of rupture length was determined as the maximum of Gaussian fit to the statistical histogram measured for over 1000 rupture events. The experimental value 31 nm is very close to the expected rupture length for covalently attached SfiI (27 nm) supporting the interpretation that specific DNA – SfiI interactions were detected using the proposed setup.

The peak with low rupture force value, 33 pN, appears in these experiments suggesting that in addition to pre-synaptic complex we were able to detect the formation of the synaptic complex. Lower value for the rupture force is explained by less loading rate in these experiments (see the dependence of the rupture force on the loading rate below). Therefore, the peak with high rupture forces corresponds to probing of pre-synaptic complexes suggesting that the rupture forces for these complexes are considerably greater than those for the synaptic complex. However, we have to consider an alternative model in which two hanging DNA duplexes form a complex with the enzyme (fig. 2f), so the dissociation of such a complex (Fig. 2g) requires large rupture force. Although, we have deliberately chosen to analyze only force curves with apparent single rupture events, one can never eliminate the possibility of double contact rupture in AFM experiments. To distinguish between two aforementioned possibilities we have performed an experiment with lower (10 fold) surface concentration of DNA duplexes on the tip. The results are shown in Fig 7. Experiments in the absence of the duplex in solution provide forces spanning over a broad range, so the distribution can be resolved into two Gaussians (blue histogram in Fig. 7). The peak for low forces corresponds to the value 38 pN and the

second peak is centered around 88 pN. After addition of free DNA to the solution large forces disappear, so the histogram is approximated by one Gaussian with a peak value 35 pN (red histogram in Fig. 7). These are the events combining synaptic and pre-synaptic complexes that we are unable to resolve, suggesting that the stability of these two complexes is quite close. It should be noted that the 10 fold decrease of the duplex concentration in the reaction mixture for the tip functionalization did not remove the probability of double rupture events. This effect could be due to relatively short polymer tether which may not provide enough flexibility, because the use of longer linker (polyT₅₀) already revealed the first peak at higher DNA concentrations (data not shown). Thus, the data obtained are in favor of the second model suggesting that probing of immobilized enzyme leads to the formation of pre-synaptic complex forming by one hanging duplex and a synaptic complex formed by two immobilized DNA duplexes.

Dynamic force spectroscopy study

In order to characterize the properties of the SfiI-DNA complex at conditions approaching to the equilibrium, we applied the dynamic force spectroscopy approach (DFS) by performing pulling experiments at various loading rates.^{17, 24} The strength of interactions within SfiI-DNA complexes was tested over a wide range of loading rates. The histograms of forces collected for each experiment were approximated by Gaussian distributions and the values corresponding to the maxima for each distribution were plotted against the logarithm of apparent loading rate. Figure 8 shows such a plot that reveals that the data points are fitted with linear plot. According to the equation 1,

$$F_R = \frac{k_B T}{x_\beta} \ln \left(\frac{v \cdot x_\beta}{k_{off} k_B T} \right) \quad (1)$$

the slope of the linear fit allows us to determine the activation energy barrier position along the direction of applied force.²⁵ The dissociation rate (k_{off}) value was obtained by extrapolating the experimental data to zero rupture force. These data along with the x_β values are assembled in Table 1. It should be noted that the rupture force dependence on the loading rate was found very similar for both pre-synaptic and synaptic SfiI-DNA complexes providing thus, similar values of x_β and k_{off} .

Spacer sequence dependence

SfiI enzyme recognizes palindromic sequence of DNA duplex (5'-GGCCNNNN[↓]NGGCC-3'). The central part of the entire DNA binding region 5 bp nonspecific spacer (-NNNN[↓]N-) is not essential for binding specificity,²⁶ and according to the crystallographic data, the protein has no direct contacts with the DNA bases inside the spacer.⁶ However, this 5 base pair central sequence affects enzymatic activity of the SfiI endonuclease.² Naturally occurring sequences are cleaved by SfiI at different rates.⁷ To get insight into the role of this part of the DNA cognate region on the complex stability, we performed force spectroscopy measurements with 3 different central 5-bp sequences. Previous studies on SfiI activity have used several different sequences.² Among them -AAAAA- and -AAACA- were used to reveal the effect of rigidity of the DNA spacer structure on the activity of the enzyme. The former sequence consists of adenines only, thus, inducing rigidity onto the DNA structure. The latter one has an oligoA sequence disrupted by a single C that was used to clarify the effect of a single base substitution. The enzymatic activity of the SfiI was reported to increase by a factor of 5 upon exchange of -AAAAA- to -AAACA- sequence.² We also compared these two sequences with -TCGAG-spacer of a random sequence. The complexes formed by each duplex design were studied by the dynamic force spectroscopy to obtain kinetic parameters for the complexes as described above. The results of the DFS analysis are shown in Figure 8. For each complex, the data points for the most probable rupture forces fall on straight lines that are different for each sequence.

If the slopes for these linear plots are quite similar, the lines shift relative to each other suggesting that the linker sequence influences dissociation rates of the complex. The effect is rather strong, so that the stability of the complexes for the sequences analyzed varies by an order of magnitude. For example, replacement of the spacer sequence from (3'-TCGAG-5') to (3'-AAAAA-5') results in the increase of dissociation rate from 38 s^{-1} to 248 s^{-1} . Table 1 summarizes dissociation rates, k_{off} , as well as the energy barrier positions, x_{β} , for all three studied nonspecific spacer sequences (-NNNNN-).

Discussion

Dynamic force measurements using our experimental strategies permitted us to reconstruct the energy landscape of the dissociation reaction of SfiI-DNA complex which is not accessible by any of the other techniques applied to this system so far. The data points for the most probable unbinding force fall on a single linear plot, suggesting that the dissociation path of the complexes has a single barrier on the energy landscape. Note that single barrier mechanisms were observed for DNA-protein systems such as restriction enzymes BsoBI, XhoI²⁷ and a binding domain of PhoR transcription activator.²⁸ Our data obtained for the range of forces starting with as low as 20 pN (the lowest values for typical AFM force spectroscopy experiments) comply with the single-barrier model. Another parameter that can be determined from the DFS analysis is the height of the energy barrier, $\Delta G^{\#}$, which determines the dissociation rate and, therefore, lifetime of a complex according to the following equation:²⁹

$$k_{\text{off}}^0 = \frac{k_B T}{h} \exp\left(-\frac{\Delta G^{\#}}{k_B T}\right) \quad (2)$$

The analyses performed for all three duplexes differed in the spacer sequence provided the following $\Delta G^{\#}$ values: 15.3 kcal/mol for (3'-TCGAG-5'), 14.2 kcal/mol for (3'-AAAAA-5') sequence, and $\Delta G^{\#}$ =14.9 kcal/mol for the intermediately stable (-AAACA-) sequence. These activation energy differences translated to a substantial difference in the dissociation off-rates (Table 1), which is the major effect of the 5 bp spacer sequence on the complex stability.

For the data analysis, we used the plot of most probable rupture forces obtained from the Gaussian fit of force histograms versus the values of the apparent loading rate (retraction velocity multiplied by spring constant of the AFM tip). This data treatment assumes that the force loading rate dF/dt is constant. However, this assumption may not be valid if a flexible linker is used for tethering the molecules. The utilization of the polymer linkers is a necessity for it reduces ambiguity of data analysis. First, the specific rupture events are moved away from strong non-specific adhesion peak. Second, it greatly reduces the probability of measuring multiple interactions between the tip and the surface. A traditional tether is PEG, a flexible polymer with relatively small persistence length, P , (ca. 0.3 nm) and it was shown that a spring constant of such a linker can contribute to the non-linearity of the dependence of applied force on the separation.³⁰ In our experiments, we used a linker consisting primarily of single stranded oligo dT chain. This polymer is considerably stiffer than PEG and characterized at the ionic conditions used at the persistence length ca. 3 nm.³¹ The spring constants of polymeric molecules fall as $(P)^{-2}$,³² therefore an anticipated spring constant for (dT)_n linkers is 100 times less than that of PEG suggesting that this polymer extends at very low applied forces. Single stranded DNA with no secondary structure extends at forces ca. 4 pN,³³ that is considerably less than minimal 13 pN noise level for AFM cantilevers used in this work.³⁴ The linker used in this study is a complex one containing short polyethyleneglycol (5 monomeric units), single stranded poly-dT (dT₁₀ or dT₅₀) and double stranded DNA (part of the duplex that is not involved in complex formation with SfiI). To test that the estimates are valid, we performed experiments with (dT)_n linkers of two different lengths, 10 and 50 monomer units. The results in Figure S3 (supporting information) show that despite the 5 times difference in length between dT₁₀ and dT₅₀ linkers they did not display substantial difference in the dependence

of the rupture forces on the apparent loading rate. This result characterizes poly-dT linker as a very good candidate for the force spectroscopy measurements when tethering via flexible linker is required.

There are two common methods used in the literature for data analysis and obtaining energy barrier position, x_β , and off-rate values, k_{off} . One method uses the dependence of rupture force on probe velocity (apparent loading rate).^{30; 35; 36; 37; 38} An alternative approach has been proposed that overcomes the deficit of the assumption of linear force/time dependence used in derivation of Equation 1. This approach uses so-called effective loading rate instead of apparent loading rate.^{13; 14; 39; 40} The effective loading rate is determined experimentally from the slope of the force versus time immediately prior the rupture point (as shown in Figure S1 – supporting information). We have compared both methods of data analysis for one of the sequences (-GGCCTCGAGGGCC-). The comparison of the rupture force dependence on apparent-vs.-effective loading rate reveals that the two methods provide different values of the definable parameters. The energy barrier position is slightly underestimated and off-rate is overestimated by factor of 2.7 when effective loading rate was used (Figure S2 –in supporting information) as compared to the use of apparent loading rate.

We have found that the central 5 bp region in the recognition site sequences (5'-GGCCNNNN↓NGGCC-3') between specific regions (GGCC) influences the stability of the SfiI-DNA complex. The recognition sequence with a linker (-TCGAG-) resulted in the formation of the most stable complex with $k_{off}=38\text{ s}^{-1}$, while polyA spacer sequence (-AAAAA-) produced the least stable complex with large value of $k_{off}=248\text{ s}^{-1}$ (Table 1). Disruption of the consecutive adenine run by a single cytosine in the middle gives rise to a more flexible linker; as a result a smaller value of dissociation rate, $k_{off}=70\text{ s}^{-1}$, was measured. These findings are in line with the data on the spacer effect on SfiI catalytic activity. For example, SfiI cleaves (-GGCCAAACAGGCC-) sequence ~5 times faster than the spacer sequence containing only adenines (-GGCCAAAAGGCC-).² A hierarchy of the nonspecific 5 base pair spacer sequence has been previously related to the flexibility of the spacer.² DNA sequences which contain A-tract (with ≥ 4 consecutive adenines) are known to be rigid.⁴¹ Indeed, according to the crystallographic data, DNA is slightly bent;⁶ therefore DNA with more flexible spacers should be better substrates for SfiI enzymatic action.² The same tendency of the sequence influence on the cleavage rates and complex dissociation rates permits us to conclude that there is an intimate relation between SfiI enzymatic activity and stability of SfiI-DNA complex.

In principle, the SfiI-DNA complex can dissociate by different pathways under applied external force, breaking the DNA-protein contacts or possibly rupture of the protein tetramer. What is the weakest link? There are two major indirect pieces of evidence supporting the hypothesis that the DNA-protein contacts are broken upon the complex dissociation. First, if the protein-protein contacts were the weakest link, after the first pulling, the protein would remain bound to the tip. Therefore, the formation of the complex upon the second approach cycle would be unsuccessful until the protein dissociates from the tip. The delay would be essential in experiments with fast pulling rates. We did not notice such delays. Second, different spacer sequences produce different off-rates. According to the crystallographic data, there is no direct contact of the spacer with the protein and thus effect of this sequence on the protein tetramer stability should be negligible. However, the difference in the values of the off-rates of almost one order of magnitude for various spacer sequences suggests substantial effect of the spacer nature indicating that the bonds between DNA and SfiI are ruptured rather than protein – protein contacts.

The results on the off-rate measurements for the SfiI-DNA complex suggest that protein dissociates from the DNA template relatively fast with a characteristic time ca. 30 ms. At the

same time, AFM topography data⁵ show that the synaptic complex is quite stable, for instance, a high yield of complexes is observed at almost equimolar protein/DNA ratios. One may suggest that the high off-rate values were obtained due to the extrapolation from relatively high loading rates typically used in the AFM force spectroscopy experiments. For example, the use of biomembrane force probe capable of measuring small forces for biotin-streptavidin complexes revealed a component in the force vs. loading rate dependence with a small slope undetectable with AFM.¹⁷ However, our recent measurements of the off-rate are constant with the use of the FCS approach, provided the dissociation time 9 ms that is similar time range for the values obtained from the DFS experiments. These data suggest that the highly dynamic behavior of the SfiI-DNA complexes is a characteristic feature of this system rather than an artifact of the DFS technique.

To reconcile all observations, we propose the following model for the SfiI-DNA complex: The protein forms a dynamic complex in which it transiently dissociates from the DNA, but does it in such a way that it remains in a close vicinity to the recognition site. For example, the protein dissociates from one half of its discontinuous recognition sequence, but remains bound to another half. It binds again to the site, but may leave the second half of the recognition region. In other words, the DNA helix behaves as a roller swing that intermittently makes contacts with one or another DNA binding site. The stiffer the spacer between these regions, the complex lifetime shortens. Indeed, according to the crystallographic data, DNA is bent between the two -GGCC- specific recognition regions, therefore stiff duplexes between the binding sites should decrease the complex stability. This is exactly what we observed in the experiments with A5 tract as a spacer that has elevated stiffness compared to other tested sequences. However, direct experiments are needed to test this model and these experiments are in progress.

The synaptic SfiI-DNA complex has all features characteristic for the site specific DNA recombination complexes formed by various proteins. Interaction of these proteins with DNA should be dynamic to facilitate accomplishing their major function - to cut and rearrange DNA molecules. Therefore the approaches used in this paper will be able to analyze such dynamic systems and provide a quantitative characterization of the biologically important nucleoprotein complexes.

Materials and methods

Preparation of oligonucleotides

DNA oligonucleotides with flexible single-stranded polyT linker (either 10bp or 50bp), containing 13bp recognition site (3'-GGCCNNNNNGGCC-5') and thiol modification at 5' end were purchased from Integrated DNA Technologies, Inc. as single stranded complements. The oligonucleotides were dissolved in dd water and 20 μ l aliquots were stored at -80°C until use. Complementary oligonucleotides were annealed by heating to 98°C , followed by a slow cooling to room temperature. To reduce protected thiol groups of the oligonucleotides, the duplexes were treated with 10 mM buffered solution (10 mM HEPES, 50 mM NaCl, pH 7.0) of TCEP-hydrochloride (Tris(2-carboxyethyl)phosphine) at 25°C for 10 min.

Tip and surface modification

Silicon nitride (Si_3N_4) AFM tips were initially washed in ethanol by immersion for 30 min and then activated by 30 min UV treatment. Activated tips and freshly cleaved mica surface were treated with 167 μM solution of maleimide-silatrane for 3 hours followed by rinsing with dd water. Maleimide functionalized AFM tips and mica were incubated for 1 hour respectively in 10 μM and 80 μM of double stranded DNA. For covalent attachment of SfiI protein, 8 nM solution of the protein (low BSA content, New England Biolabs) was treated with TCEP-

hydrochloride and then maleimide functionalized mica surface was incubated with this solution for 1 hour. After washing with HEPES buffer solution (10 mM HEPES, 50 mM NaCl, pH 7.0), unreacted maleimide moieties were quenched with buffered 10 mM β -mercaptoethanol solution by 10 min. treatment at room temperature. The modified tips and mica were washed with 10 mM HEPES, 50 mM NaCl, pH 7.0 and stored in the same buffer until use.

AFM measurements

Force-distance measurements were performed in buffer solution (10 mM HEPES, 50 mM NaCl, 2 mM CaCl_2 , pH 7.0) at room temperature by Molecular Force Probe 3D system (Asylum Research, Santa Barbara, CA). The ramp size used was 300 nm with various loading rates. An application force was kept at low value (100 pN). Silicon nitride cantilevers with nominal values of spring constants in the range of 0.04–0.07 N/m were used. Spring constants for each cantilever were obtained using thermal method with the MFP-3D instrument. For control experiments, the SfiI protein was blocked by injection of a 2000 molar excess of DNA competitor. Approach and retraction velocities were the same (150 nm/s) except for a set of dynamic force measurements in which approach velocity was fixed at 600 nm/s and retraction velocity was varied between 50 and 10000 nm/s corresponding to the values of apparent loading rates (cantilever spring constant multiplied by retraction velocity) between 2.1×10^3 and 6.3×10^5 pN/s. The elastic response of a linker to applied force was analyzed by software package (Igor Pro 5.03) using worm-like-chain (WLC) approximation. The effective loading rate values were determined from the slope of the force curve immediately prior (~ 1 nm) the position of the rupture point. The end-to-end distances (contour lengths) were determined from the WLC fit of experimental force-distance curves. The most probable rupture force was obtained from the maximum of the Gaussian fit to the force distribution combined in a statistical histogram. Normally the rupture forces of more than 500 rupture events were compiled in force distribution histograms. The maximum of Gaussian fit to each histogram at any given loading rate defines the most probable rupture force, F^R , which is described by the equation 1.⁴²

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic representation of the experiment for studying synaptic SfiI-DNA complexes. (a) oligoduplexes were covalently attached to both AFM tip and mica surface. (b) buffer solution of SfiI was added between tip and the surface resulting in the formation of synaptic complex after the tip approaches the surface. (c) application of pulling force results in the rupture of the complex.

Figure 2.

Schematic representation of the experiment with protein immobilization approach for studying presynaptic SfiI-DNA complex. (a) AFM tip was modified with oligoduplexes and SfiI was immobilized on the surface. (b) Approach the tip to the surface to form the complex. (c) Application of pulling force results in the rupture of the presynaptic complex. (d) High concentration of target molecules results in the rupture of multiple contacts. (e, f) Addition of free DNA duplex to the solution increases probability of single complex rupture due to blocking active SfiI sites.

Figure 3.

A typical force-distance curve obtained for the interactions between SfiI and DNA oligonucleotide: 1) the start point of the tip retraction from surface, the force curve in this region reflects the nonspecific adhesion forces between surface and the tip; 2) the region is indicative of polymer linker stretching, this portion of the curve is fitted with worm-like chain approximation (solid red line); 3) the abrupt jump in the force curve is typical of specific bond rupture. The inset shows the rupture force histogram obtained for the synaptic complex at 150 nm/s retraction velocity (~ 9.5 nN/s apparent loading rate).

Figure 4.

Distributions of contour lengths obtained from WLC fitting for different linkers: (a) 10bp polyT linker for both oligonucleotides on the tip and on the surface producing 36 nm of contour length; (b) 50bp polyT linker for oligonucleotide on the tip and 10bp polyT linker for oligonucleotide on the surface (50 nm); (c) 10bp polyT + PEG (MW=3400 g/mol) linker for oligonucleotide on the tip and 10bp polyT linker for the oligonucleotide on the surface (61 ± 5 nm).

Figure 5.

A typical force-distance curve obtained for the interactions between DNA oligonucleotide and surface immobilized SfiI. The inset shows rupture force histogram obtained for the secondary complex at 150 nm/s retraction velocity ($\sim 9.5 \text{ nN/s}$ apparent loading rate).

Figure 6.

(a) Rupture of SfiI-DNA complexes in the experiments with covalently attached protein and free oligoduplexes in solution (GGCCTCGAGGGCC – recognition sequence) at 150 nm/s retraction velocity (~ 6.5 nN/s apparent loading rate). Two distinct populations of forces are evident (see text for details). (b) Distributions of contour lengths obtained from WLC fitting in the experimental setup with surface immobilized SfiI and oligoduplex with (dT)₁₀ linker on the tip (27 nm).

Figure 7.

The rupture force distribution at low concentration of DNA on the tip. Two force populations are observed (blue histogram): the first peak corresponds to single complex rupture, the second peak corresponds to the situation when two complexes are ruptured simultaneously. Addition of free DNA duplex to the solution completely eliminated the possibility of double contact formation (red histogram).

Figure 8.

Dynamic force spectra of SfiI-DNA complexes for three spacer sequences: -TCGAG- = (blue); -AAACA- = (red); -AAAAA- = (green).

Table 1

Experimental values of k_{off} and x_{β} for SfiI-DNA complex obtained using dynamic force spectroscopy. Standard errors of the experimentally determined values are given in parentheses.

Recognition sequence	k_{off} s ⁻¹	x_{β} Å
5'GGCCT CGA^GGGCC CCGGA^GCT CCCGG 3'	38(8)	1.8(0.2)
5'GGCCA AAC^AGGGCC CCGGT^TTG TCCCGG 3'	70(11)	1.9(0.1)
5'GGCCA AAA^AGGCC CCGGT^TT T TCCGG 3'	248(79)	1.9(0.2)