Real-Time Kinetics Measurements of Protein Induced Conformational Changes in DNA

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***Abstract*- We combine electrical orientation of surface bound dsDNA probes with an optical technique for kinetic measurement of protein-induced conformational changes in DNA. The effects of probe surface density and protein variation are investigated.**

I. Introduction

Integration Host Factor (IHF) is an abundant protein that functions in several processes involving higher-order protein-DNA complexes, e.g., in replication, transcription, regulation, and a variety of site specific recombination systems. The steady-state IHF-DNA interaction is well characterized and, based on X-Ray crystallography, NMR, and AFM, it is well understood that IHF binds to DNA with high sequence specificity and bends the DNA 160° [1]. Micro-arrayed detection schemes are currently the primary means for high throughput measurements of protein-DNA interactions and the kinetics of protein interactions with surface bound probes have, thus far, remained largely unexplored. Here we demonstrate a platform to measure the kinetics of IHF-induced bending of surface bound DNA probes.

II. Experiment

2mm diameter Au electrodes adjacent to Pt counterelectrodes were patterned on a sapphire wafer. 80 base pair long double stranded DNA oligonucleotides (dsDNA) were functionalized with a thiol on the proximal end and with a Cy3 fluorophore on the distal end and end-grafted on the gold electrode inside a fluidic channel. Two different probe sequences were designed and immobilized on separate electrodes: Probe 1 contains the IHF H' binding site, derived from the phage lambda attP sequence, near the proximal end and Probe 2 contains the H' binding site near the distal end. A microscope in reflected dark field mode (Olympus BX-RLA2) was used for the probe illumination (CW DPSS laser, 532nm, Newport Spectra Physics, Pro Millennia) and the fluorescence light detection. We filter reflected light from the laser illumination using a long wave pass filter (550±5 nm, Newport, 10LWF-550) and a band pass filter (Semrock, FF01-582/75-25). The probe fluorescent emission intensity (Cy3, peak emission ~570 nm) is detected by a cooled photomultiplier (Hamamatsu, Photosensor Modules H7422) operating in the single-photon-counting mode.

The efficiency of non-radiative energy transfer (ET) between the fluorophores and surface plasmons in gold follows the distance dependency, $ET \propto d^{-3}$, where d is the distance between the fluorophore and the gold surface. This allows for real time observation of the emitter – gold distance through measurement of the fluorescent intensity [2]. As the contour length of the tethered dsDNA is significantly less than the persistence length (l_c~27 nm, l_p~ 50 nm), the conformation is that of a rigid rod and orientation of the dsDNA probes, relative to the gold surface, can be inferred [3, 4].

The orientation of immobilized dsDNA is controlled through a voltage potential applied to the gold electrode with respect to the platinum counter electrode. This results in the formation of an ionic double layer at the electrode surfaces and screens the electric field so that it is confined to a few nanometers from the electrode surface. For low potentials the voltage potential, as a function of distance from the electrode, reduces to the *Debye- Hückel* equation, $\psi \sim \psi_0 e^{-x/l_d}$, where the Debye length appears as the characteristic decay length of the potential [5]. A large electric field results a few Debye lengths from the gold surface and exerts an electrostatic force on immobilized dsDNA that overcomes the Brownian motion, resulting in uniform alignment of the dsDNA in the electric field [6]. Due to the rapid decay of the electric field, the electrostatic force on the dsDNA and other charged molecules (such as charged proteins) becomes negligible at a few Debye lengths from the gold surface [6]. In a 60 mM monovalent salt solution the Debye length is ~1.2 nm. For a more detailed description and depiction of the setup see [4].

III. RESULTS & DISCUSSION

The observed binding time constants, on the order of a few minutes, are several orders of magnitude higher than what was reported for specific IHF-dsDNA binding in free solution, which are on the order of hundreds of milliseconds [7]. This suggests that the steric hindrance of densely immobilized dsDNA alters the kinetics of IHF-DNA binding. To test the effect of dsDNA probe surface density on protein binding, we prepared dsDNA layers of varying surface densities by immobilizing a densely packed layer (~10^{12} cm^{-2}) and reducing the surface density through controlled electrical desorption. We determine surface density qualitatively by monitoring the fluorescent signal and the switching efficiency. The switching efficiency corresponds to the difference in the fluorescent intensity between DNA in lying (positive applied potential) and standing (negative applied potential) orientation. This is highly correlated to surface layer density [4]. As the DNA layer is desorbed the switching efficiency initially increases due to reduced steric hindrance between adjacent DNA probes until it peaks and drops due to an overall decrease in fluorescent intensity against a constant background (see [8] for a detailed description). As Figure 1a shows, reducing the oligonucleotide surface density increases the IHF-oligonucleotide binding rate by more than twofold.

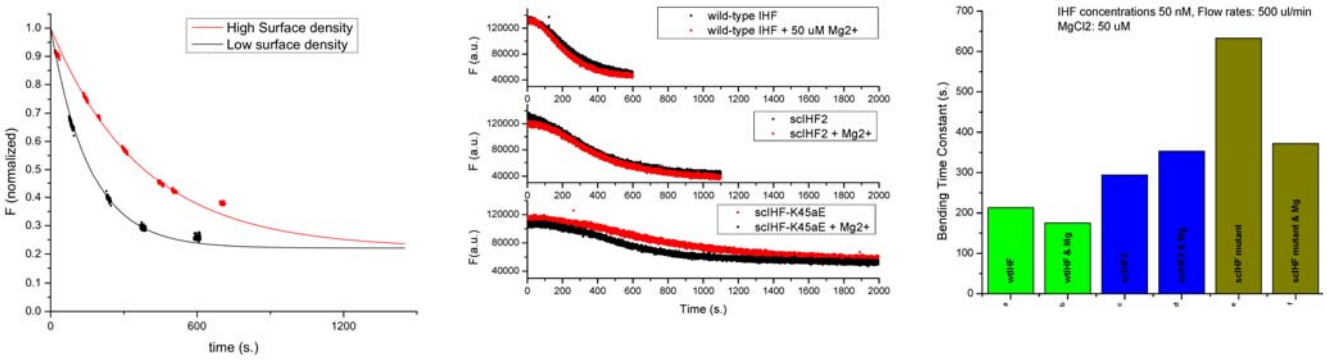

Figure 1a: Normalized fluorescence of Cy3 labeled oligonucleotide probes containing the IHF binding sequence near the proximal end, measured during addition of 50 nM wild-type IHF. The total degree of bending is equal for both sequences but the binding rate varies greatly; a single exponential decay fit gives a time constant, τ = 158 seconds for the low surface density oligonucleotide layer versus τ = 374 seconds. **Figure 1b:** Overlaid real-time measurements of fluorescence during addition of wild-type IHF, scIHF2, and scIHF-K45αE with and without 50 uM Mg^{2+}. **Figure 1c:** Comparison of binding rates indicates that wild-type IHF is the fastest binder and scIHF-K45αE is the slowest while addition of 50 uM Mg^{2+} does not influence binding for wild-type IHF and scIHF2 but leads to a 60% increase in the binding rate for scIHF- K45αE induced bending.

Due to its high impact on protein binding kinetics, it is important to have high repeatability of the dsDNA layer surface density. One way to achieve this is through regeneration of the oligonucleotide layer to its pre-IHF-bound state by denaturing and washing away bound protein. We flow 0.4% SDS in 0.6 M NaCl to denature and remove IHF bound to dsDNA probes. The fluorescence intensity recovers to approximately 90% the pre-IHF bound levels and a near complete regeneration of the oligonucleotide layer is achieved by flowing a 100 nM complementary oligonucleotide solution to re-hybridize DNA. We measured fluorescent intensity during successive binding and removal of wild-type IHF and found binding kinetics to be highly repeatable. Measurements were made for wild-type IHF addition at flow rates of 50, 500 and 1500 ul/min and a significant change in binding kinetics is noted when the flow rate is increased from 50 to 500 ul/min during IHF addition. For flow rates of 500 and 1500 ul/min the binding kinetics are nearly identical, indicating a reaction-limited regime. Consequently all subsequent measurements were made using a 500 ul/min flow rate. We repeated this cycle of IHF-oligonucleotide binding followed by protein removal and dsDNA layer regeneration up to 15 times with highly repeatable IHF induced bending followed by regeneration and signal recovery. One drawback appears to be a gradual increase in the protein binding rate and small drop in fluorescence level, indicating a slow decrease in dsDNA layer surface density following each regeneration step.

We investigate the bending efficacy of three IHF types: 1) wild-type IHF, 2) scIHF2, an engineered single chain IHF that is functional in mammalian cells and 3) scIHF-K45αE, a variant of scIHF2 that carries glutamate instead of lysine at position 45 of the α subunit (for a detailed description of the engineered proteins see [1]). DNA bending by wild-type IHF and scIHF2 were previously determined by AFM and the magnitude of bending was shown to be about 160° for both IHF types. The same experiments also show an increase in stability of the scIHF-K45αE –DNA complex and resulting increase of DNA bending, from about 90° to about 110° due to the presence of Mg^{2+} [1]. To test the effect of IHF types on oligonucleotide bending we utilized the protocol described above to regenerate the dsDNA layer and observed bending with the addition of the different IHF types, with and without the presence of Mg^{2+}. The results are summarized in Figure 1b and 1c. We observe that the magnitude of bending is approximately equal for wild-type IHF and scIHF2 but reduced for scIHF-K45αE. Wild-type IHF induced binding is the fastest and scIHF-K45αE is the slowest while Mg^{2+} does not affect the magnitude or the rate of binding for wild-type IHF and scIHF2. However, addition of 50 uM Mg^{2+} leads to a 60% increase in the binding rate for scIHF-K45αE.

IV. Conclusions

We demonstrate a novel platform to detect the kinetics of IHF induced conformational changes in DNA *in-situ* and in real-time. The on chip probe immobilization and optical detection make this technique suitable for micro-arrayed high-throughput applications.

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